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Βιολογικοί δείκτες βασισμένοι στην πρωτεΐνη LRRK2 στη νόσο Πάρκινσον

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Βιοχημικός

«ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ»

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«Η υλοποίηση της διδακτορικής διατριβής συγχρηματοδοτήθηκε από την Ελλάδα και την Ευρωπαϊκή Ένωση (Ευρωπαϊκό Κοινωνικό Ταμείο) μέσω του Επιχειρησιακού Προγράμματος «Ανάπτυξη Ανθρώπινου Δυναμικού, Εκπαίδευση και Δια Βίου Μάθηση», 2014-2020, στο πλαίσιο της Πράξης «Ενίσχυση του ανθρώπινου δυναμικού μέσω της υλοποίησης διδακτορικής έρευνας- Υποδράση 2: Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ σε υποψηφίους διδάκτορες των ΑΕΙ της Ελλάδας».

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ΟΡΚΟΣ ΤΟΥ ΙΠΠΟΚΡΑΤΗ

Ὅμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγίαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, Ἱστορας ποιεύμενος, ἐπιτελέα ποιήσιν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὄρκον τόνδε καὶ συγγραφὴν τήνδε.

Ἠγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτησιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηρίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ αὐτοῦ ἀδελφοῖς ἴσον ἐπικρινέειν ἄρρεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἣν χρηρίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ συγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἀπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοις τε καὶ ὠρκισμένοις νόμῳ ἰητρικῷ, ἅλλω δὲ οὐδενί.

Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἶρξιν.

Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑψηγήσομαι συμβουλίην τοιήνδε. Ὅμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἀγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν.

Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτησιν ἀνδράσι πρήξιος τῆσδε.

Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἐκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπὶ τε γυναικείων σωμάτων καὶ ἀνδρώων, ἐλευθέρων τε καὶ δούλων.

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Petropoulou-Vathi L, Simitsi A, Valkimadi P-E, Kedariti M, Dimitrakopoulos L, Koros C, et al. Distinct profiles of LRRK2 activation and Rab GTPase phosphorylation in clinical samples from different PD cohorts. *npj Parkinsons Dis.* 8, 73 (2022).

Polissidis A, **Petropoulou-Vathi L**, Nakos-Bimpos M, Rideout HJ. The Future of Targeted Gene-Based Treatment Strategies and Biomarkers in Parkinson's Disease. *Biomolecules.* 2020;10(6).

Memou A, Dimitrakopoulos L, Kedariti M, Kentros M, Lamprou A, **Petropoulou-Vathi L**, et al. Defining (and blocking) neuronal death in Parkinson's disease: Does it matter what we call it? *Brain Research.* 2021;1771:147639.

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ΕΥΧΑΡΙΣΤΙΕΣ

Για την ολοκλήρωση της συγκεκριμένης διδακτορικής διατριβής χρειάστηκε η συμβολή και η βοήθεια πολλών ανθρώπων. Πρώτον απ' όλους, οφείλω να ευχαριστήσω τον επιστημονικό μου υπεύθυνο, Δρ. Hardy Rideout, ο οποίος ήταν ο πρώτος που με εμπιστεύτηκε σε αυτό το βήμα και έπαιξε καθοριστικό ρόλο στην εξέλιξή μου. Ήταν δίπλα μου από την πρώτη στιγμή και εκτιμώ ότι ακόμα και σε δύσκολους καιρούς προσπαθούσε πάντα για το καλύτερο. Πέρα από την άρτια επιστημονική του καθοδήγηση και την ανεκτίμητη βοήθειά του σε όλα τα στάδια του διδακτορικού, ήξερα ότι θα είναι εκεί για να με στηρίξει σε ό,τι χρειαστώ και αυτό είναι το πιο σημαντικό από τη συνεργασία μας για έμένα.

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Στους γονείς μου...

ABBREVIATIONS

ANK: Ankyrin repeat

ARM: Armadillo repeat

A.U.: Arbitrary units

BAC: Bacterial artificial chromosome

BSA: Bovine serum albumin

CK1 α : Casein kinase 1 α

COR: C-terminal of ROC

CSF: Cerebrospinal fluid

CUMC: Columbia University Medical Centre

DA: Dopamine

DMSO: Dimethyl sulfoxide

D-PBS: Dulbecco's phosphate-buffered saline

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ERM: Ezrin/radixin/moesin

EVs: Extracellular vesicles

FADD: Fas-associated death domain

GAP: GTPase-activating protein

GEF: Guanine nucleotide exchange factor

GDI: Guanosine nucleotide dissociation inhibitor

GWAS: Genome-wide association studies

HC: Healthy control

HEK: Human embryonic kidney

HRP: Horseradish peroxidase

IFN- γ : Interferon- γ

IL-1 β : Interleukin-1 β

IP: Immunoprecipitation

iPD: Idiopathic PD

K/D: Knockdown

K/O: Knockout

LAMP1/2: Lysosomal-associated membrane protein 1/2

LC3: Microtubule-associated protein 1A/1B-light chain 3

LB: Lewy body

LEDD: Levodopa equivalent dose

LPS: Lipopolysaccharide

LRR: Leucine-rich repeat

LRRK2: Leucine-rich repeat kinase 2

MBP: Myelin basic protein

MDS-UPDRS: Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale

MoCA: Montreal Cognitive Assessment

NK: Natural killer

NLRC4: NLR family CARD domain-containing protein 4

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PD: Parkinson's disease

PKA: Protein kinase A

PP1: Protein phosphatase 1

PP2A: Protein phosphatase-2A

PPM1H: Protein phosphatase Mg²⁺/Mn²⁺ dependent 1H

PTM: Post-translational modification

rAAV: Recombinant adeno-associated virus

ROC: Ras-like GTPase domain

ROCO: Ras of complex proteins

ROS: Reactive oxygen species

RILPL1: Rab interacting lysosomal protein Like 1

RT: Room temperature

SA: Streptavidin

SDS-PAGE: Sodium dodecyl sulfate–polyAcrylamide gel electrophoresis

S/N: Supernatant

SNP: Single nucleotide polymorphism

SN_{pc}: Substantia nigra pars compacta

SNpr: Substantia nigra pars reticulata

STR: Striatum

TBK1: TANK-binding kinase 1

TEM: Transmission electron microscopy

TGN: Trans-Golgi network

TH+: Tyrosine hydroxylase positive

TLR4: Toll-like receptor 4

TNF- α : Tumour necrosis factor

VMB: Ventral midbrain

VTA: Ventral tegmental area

WB: Western immunoblotting

WT: Wild-type

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ΠΕΡΙΛΗΨΗ

Το κεντρικό θέμα της συγκεκριμένης διδακτορικής διατριβής ήταν η αξιολόγηση των επιπέδων έκφρασης και δράσης της πρωτεΐνης LRRK2 σε διαφορετικά βιολογικά δείγματα από ασθενείς με νόσο Πάρκινσον. Παρότι έχει υπάρξει σημαντική πρόοδος τα τελευταία χρόνια στην εύρεση βιοδεικτών στο πεδίο, υπάρχει ακόμα η ανάγκη για ανάπτυξη αξιόπιστων μεθόδων ανίχνευσής τους. Υπάρχουν στοιχεία που υποδεικνύουν ότι η δράση της συγκεκριμένης πρωτεΐνης παίζει κεντρικό ρόλο στην φλεγμονώδη αντίδραση που παρατηρείται τόσο στις κληρονομικές, όσο και στις ιδιοπαθείς μορφές της νόσου. Για αυτό το λόγο σε αυτή τη μελέτη συμπεριλήφθηκαν συμμετέχοντες με διαφορετικά γενετικά υπόβαθρα. Γνωρίζουμε από πειραματικά μοντέλα ότι η πιο συχνή μετάλλαξη (G2019S) στο γονίδιο που κωδικοποιεί την LRRK2 προκαλεί αύξηση της δράσης κινάσης, η οποία θεωρείται τοξική για τα κύτταρα. Έτσι, ασθενείς με τη συγκεκριμένη μετάλλαξη καθώς και υγιείς φορείς αυτής συμπεριλήφθηκαν στη μελέτη και τα επίπεδα και η δράση της LRRK2 συγκρίθηκαν με αυτά από ασθενείς με την ιδιοπαθή μορφή της νόσου καθώς και με υγιείς συμμετέχοντες από δείγματα κυττάρων αίματος του ανοσοποιητικού και ούρων. Στην έρευνα πήραν μέρος επίσης και ασθενείς που φέρουν τη μετάλλαξη A53T στο γονίδιο SNCA το οποίο κωδικοποιεί την α-συνουκλεΐνη, πρωτεΐνη με κεντρικό ρόλο στην παθογένεια της νόσου. Η συμπερίληψη αυτών των ασθενών έγινε καθώς μελέτες έχουν δείξει ότι η δράση της LRRK2 παίζει σημαντικό ρόλο στην παθογένεια της νόσου που είναι συνδεδεμένη με την α-συνουκλεΐνη. Η παρεμπόδιση της δράσης κινάσης της LRRK2, μεσολαβούμενη από αυτόνομους και μη αυτόνομους κυτταρικούς μηχανισμούς, βελτιώνει την ντοπαμινεργική νευρωνική επιβίωση, μεταβάλλει τις ανοσοποιητικές κυτταρικές αντιδράσεις και ρυθμίζει την παθολογική συσσωμάτωση της α-συνουκλεΐνης ως αποτέλεσμα της υπερμεταγραφής ή της έκθεσης σε εξωκυτταρικά είδη α-συνουκλεΐνης. Σύμφωνα με τα παραπάνω, είναι σημαντικό να εξεταστεί αν παρατηρούνται διαφορές στην έκφραση και στη δράση της LRRK2 σε αυτές τις ξεχωριστές ομάδες ασθενών ώστε αυτές οι ενδεχόμενες διαφορές να χρησιμοποιηθούν ως βιοδείκτες για τη διάγνωση και την παρακολούθηση των ασθενών καθώς και για την διαφοροποίηση μεταξύ των διαφορετικών μορφών της νόσου. Τέλος, μπορεί να βοηθήσουν και σε κλινικές δοκιμές για την συμπερίληψη/αποκλεισμό αναστολέων της δράσης της LRRK2. Το ενδιαφέρον της συγκεκριμένης διατριβής αφορούσε κυρίως την μελέτη διαφόρων πρωτεϊνών στο μονοπάτι σηματοδότησης της LRRK2, όπως η φωσφορυλίωση της ίδιας της LRRK2 καθώς και υποστρωμάτων της όπως πρωτεΐνες που ανήκουν στην οικογένεια Rab

GTPase. Παρότι έχει υπάρξει σημαντική πρόοδος τα τελευταία χρόνια στην εύρεση βιοδεικτών στο πεδίο, υπάρχει ακόμα η ανάγκη για ανάπτυξη αξιόπιστων μεθόδων ανίχνευσής τους. Πιστεύεται ότι τα ευρήματα της συγκεκριμένης μελέτης θα συμβάλλουν στο πεδίο για την ανάπτυξη ευαίσθητων τεχνικών προς εύρεση βιοδεικτών που βασίζονται στην πρωτεΐνη LRRK2 και στην περαιτέρω κατανόηση της νόσου.

SUMMARY

The central question of this PhD project was whether it is possible to reliably detect changes in the levels or activity of a protein called leucine rich repeat kinase 2 (LRRK2) in different biofluid samples from people affected by Parkinson's disease (PD). There is considerable evidence now that LRRK2 activity plays a central role in the type of inflammatory response often seen in the different forms of familial and idiopathic PD (iPD), and for this reason participants of variant genetic backgrounds were studied here. It is known from experimental models that the most common LRRK2 mutation (G2019S) increases its kinase activity and this gain of function is considered toxic for cells; Consequently, affected and non-affected G2019S carriers were recruited and levels and activity of LRRK2 were compared to those of iPD patients as well as healthy controls (HC) in blood immune cells and urine biofluids. In addition, there is evidence indicating the importance of LRRK2 activity in mediating pathology related to α -synuclein, a central protein in PD pathogenesis, therefore; carriers of a mutation in the *SNCA* gene (A53T) that encodes α -synuclein, were also included. Mediated by both cell autonomous and non-cell autonomous mechanisms, the inhibition of LRRK2 kinase activity improves dopamine neuron survival, alters immune cell responses, and modulates pathological α -synuclein aggregation in response to overexpression or exposure to extracellular α -synuclein species. These lines of evidence suggest that it may be possible to detect changes in the activity of LRRK2 in samples from multiple forms of PD; and if this is the case, then it will be a powerful tool to be used as a biomarker in the staging of PD, as well as tracking its progression in patients who have already been diagnosed. Finally, such tests may be possible to discriminate between the different forms of PD and can also be utilised in clinical trials of new drugs being designed to inhibit the activity of LRRK2, to show that the drugs are having the desired effects on LRRK2. The focus of this study concerns several key proteins in the LRRK2 signalling pathway; including phosphorylation of LRRK2 itself as well as downstream substrates, such as the Rab GTPase family. While the field has made great advances in biomarker development for some targets important for PD, there is still a great need for assays measuring the activation of other signalling pathways. Our findings set the stage for further development of assays in the LRRK2 pathway and the establishment of sensitive signatures of LRRK2 activation in multiplexed immune-assays for better understanding of the disease.

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease, globally, following Alzheimer's disease (AD). While the majority of cases are idiopathic, with no known genetic cause; approximately 10% of cases can be traced to pathogenic mutations in various genes transmitted in both autosomal dominant as well as recessive manners. Among these, G2019S mutation in the gene encoding LRRK2 protein is the most common familial cause, however; there is additional evidence implicating LRRK2 activity or dysfunction in idiopathic PD (iPD). Furthermore, multiple lines of evidence show that LRRK2 plays a major role in regulating PD pathology related to α -synuclein protein, by a yet unknown mechanism. Potentially, its kinase activity could be a key event in the accumulation and aggregation of proteins within degenerating neurons. As LRRK2 function has been linked to many cellular events that contribute to PD pathogenesis, assays quantifying its activation in the context of the disease are critically needed. Like for many other neurodegenerative disorders, a standardized fluid biomarker that defines specific stages of PD is lacking. This work describes a multi-time point clinical study of two different cohorts with distinct genetic status that represents the first direct comparison of LRRK2 levels, its phosphorylation and *in vitro* kinase activity, using high throughput assays in multiple biofluids collected from the same individual of different PD groups to interrogate potential biomarkers.

An exacerbated immune response is very commonly observed in PD and LRRK2 is highly expressed in peripheral immune cells, suggesting a role in the regulation of the inflammatory process. There are no other studies, up to date, to examine aspects of LRRK2 in different immune cell types from the same individual. Additionally, LRRK2 has never been studied in biofluids coming from PD patients with a mutation in α -synuclein. Thus; the primary focus of this work was to study LRRK2 levels and intrinsic kinase activity in PBMCs and CD14⁺ monocytes isolated from blood of iPD, G2019S PD and PD patients conferring the A53T mutation in the gene encoding for α -synuclein. Phosphorylation of Rab GTPase LRRK2 endogenous substrates was also assessed as an indication of enzymatic activity. Additionally, as an alternative biofluid source, LRRK2 activation was examined in small extracellular vesicles (EVs) isolated from urine and in neuronal EVs from plasma, as previous studies have shown that LRRK2 activity is elevated in urine and CSF EVs of G2019S PD and iPD patients. In this way, a parallel comparison between patients of different genetic backgrounds but also among different biofluid sources of the same individual is provided, allowing potential distinct regulatory mechanisms that can contribute to biomarker development to be revealed.

Results demonstrate the great difference in expression levels between enriched CD14⁺ monocytes and the mixed PBMC population, cells with high implication in the innate immune response. It was found that despite the very high levels of expression of LRRK2 in monocytes, the intrinsic activity of this pool is remarkably low compared to the normalised levels of activity from PBMCs. An alternative way of measuring LRRK2 activity is by quantifying its autophosphorylation levels at Ser1292 (S1292) residue, however; this post-translational modification could not be reliably detected in these cell types. Phosphorylation at S935 residue of LRRK2 has been previously shown to serve as a pharmacodynamic marker of LRRK2 kinase activity as its levels decrease with inhibitors. These levels were significantly elevated in PBMCs of iPD patients, as well as in LRRK2 PD patients, allowing discrimination between affected and non-affected carriers of the G2019S mutation. Such an effect was absent in A53T PD patients, implying distinct regulatory mechanisms. Monocytes of the same individuals failed to show such an increase in any of the groups. Levels of Rab GTPase LRRK2 substrates were also assessed in these cell types. Rab10 phosphorylation was enhanced in PBMCs of iPD patients of two independent cohorts but not in monocytes, however; there was unreliable detection of Rab29 in both cell types. This was the first study to demonstrate presence of Rab GTPases in urine EVs. There was a non-significant trend for higher Rab10 phosphorylation in iPD patients but a significant loss of Rab29 phosphorylation in the same group as well as in A53T PD. In neuronal EVs isolated from plasma, neuronal markers indicative of successful isolation could be detected, as well as the presence of LRRK2, however; the reproducibility of this isolation method needs to be improved.

The final aspect of this project was to produce and validate an ultra-sensitive multiplexed LRRK2-pathway focused ELISA-based array that is capable of quantifying multiple independent pharmacodynamic markers of LRRK2 in a single sample simultaneously. Total LRRK2 levels and its phosphorylation at S935 residue were readily detectable in CSF as well as in immune cells of a small cohort. Thus; this assay is anticipated to be used in the future to measure these LRRK2 aspects in larger scale patient groups. Until now, the LRRK2 biomarker field has relied primarily on a mixture of western immunoblot and ELISA-based techniques, all in a single-plex mode, which results in a semi-quantitative picture of LRRK2 pathway activation. Here, the critical importance of interrogating LRRK2 in multiple sample types is highlighted, as findings have revealed potential parallel regulatory pathways in different biofluids of the same individuals as well as between different patient cohorts.

INTRODUCTION

1.1 Parkinson's disease

1.1.1 General PD and clinical symptoms

Parkinson's disease (PD) affects ~1% of the general population and is the second most common neurodegenerative disorder, first described by James Parkinson over 200 years ago [1, 2]. The basal ganglia circuit of the brain, implicated in the coordination of movement, is predominantly affected, comprising basically of the striatum (STR) (caudate-putamen) and globus pallidus, interconnected with the substantia nigra (SN), the subthalamic nucleus, the pedunculopontine nucleus, the cortex and the thalamus [3]. Hence, PD is characterised primarily by motor deficits including tremor, bradykinesia, rigidity and postural instability [2, 4, 5]. There are also a number of non-motor symptoms such as fatigue, anosmia, constipation, depression, anxiety and sleep disorders, often preceding the motor phase [6]. Most common signs and symptoms of the disease are shown in figure 1. It is a progressive disorder that mostly affects the elderly population over the age of 65; symptoms intensify with time, and cognitive impairment, to the point of dementia, severe neuropsychiatric disturbances such as psychosis and autonomic dysfunction may also appear [7, 8]. Unfortunately, there is still no effective disease-modifying therapy with only a few treatments available relieving predominantly motor-related symptoms (mostly in the early phase), such as LEVO-DOPA [6]. Diagnosis is mostly clinical and can be challenging as PD is very heterogeneous with many of its features being shared with other diseases (essential tremor, other synucleinopathies, dementia with Lewy bodies etc.) [9]. Recent criteria and motor/cognitive assessment tests are shown in table 1. It is not a life-threatening disorder and life-expectancy is generally slightly lower than that of the general population, however; death may arise as a result of afflictions associated with it (falls, pulmonary aspiration, blood clots, pulmonary embolisms etc.) [10]. The cause is unknown with major risk factor being ageing, but genetics and/or environmental factors (i.e., pesticides, diabetes, stress, smoking) may also contribute [11-13]. With age, many cellular processes fail and protective mechanisms become defective leading to neurodegeneration seen in PD.

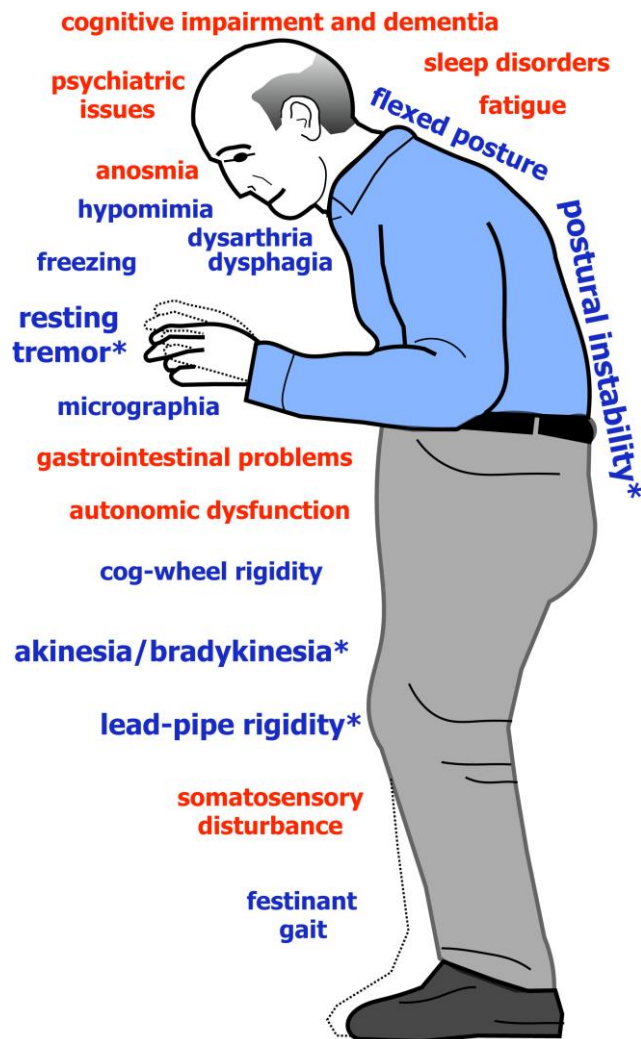


Figure 1: Signs and symptoms of Parkinson's disease. Most common motor (blue) and non-motor (red) deficits seen in PD (Adopted from *Run in the Light: Exploring Exercise and Photobiomodulation in Parkinson's Disease*).

1. *Diagnosis of parkinsonism*
 - a. Bradykinesia
Plus one of
 - b. Tremor
 - c. Rigidity
2. *Exclusion criteria*
 - a. Cerebellar abnormalities
 - b. Supranuclear gaze palsy
 - c. Diagnosis of behavioral variant of frontotemporal dementia or primary progressive aphasia within 5 years of disease onset
 - d. Parkinsonian features restricted to the lower limbs for more than 3 years
 - e. Treatment with a dopamine receptor blocker or dopamine depleting agent consistent with drug-induced parkinsonism
 - f. Absence of a response to high-dose levodopa despite at least moderate disease severity
 - g. Cortical sensory loss, clear limb ideomotor apraxia, or progressive aphasia
 - h. Normal functional imaging of the dopaminergic system ("DAT scan")
 - i. Diagnosis of alternative condition causing parkinsonism which could be causing the symptoms
3. *Supportive criteria*
 - a. Clear beneficial response to dopaminergic therapy
 - b. Presence of levodopa-induced dyskinesia
 - c. Rest tremor of a limb
 - d. The presence of either olfactory loss or cardiac sympathetic denervation on MIBG scintigraphy (although the latter is rarely done in current practice)
4. *Red flags*
 - a. Rapid progression of gait impairment leading to wheelchair use within 5 years
 - b. Absence of progression of motor symptoms over 5 years, unless related to treatment
 - c. Early bulbar dysfunction
 - d. Inspiratory respiratory dysfunction
 - e. Severe autonomic failure within the first 5 years of disease
 - f. Recurrent falls because of impaired balance within 3 years of onset
 - g. Disproportionate anterocollis or contractures within 10 years of disease onset
 - h. Absence of any of the common non-motor features despite 5 years of disease
 - i. Unexplained pyramidal signs
 - j. Bilateral symmetrical parkinsonism

Table 1: Criteria for PD diagnosis as certified by International Parkinson and Movement Disorder Society in 2015 [9]. 1. Parkinsonian symptoms include bradykinesia plus tremor or rigidity. Determination of PD as the cause of parkinsonism relies on three categories of diagnostic features: 2. Exclusion criteria, 3. Supportive criteria and 4. Red flags, as listed above.

1.1.2 Genetic forms of PD

Most cases of PD are idiopathic (sporadic in the population) with an average age of disease onset in the ~60s, however; genome-wide association studies (GWAS) have identified more than 40 risk loci for PD [14]. Several genes have been linked to familial PD (e.g., *LRRK2*, *SNCA*, *PRKN*, *PINK1*, *DJ-1*), accounting for ~10-15% that have shared characteristics but also variation depending on the PD sub-type [15-22]. Most important PD-linked genes are summarised in Table 2. In addition to these key genes, there are also genetic variants in different gene loci (e.g., *SNCA*, *LRRK2*, *GBA*, *RAB29*) that act as risk factors for developing the disease and GWAS have shown several single nucleotide polymorphisms (SNPs) across populations [23-27].

Locus	Chromosome	Gene	Name of protein	Protein function	Pathology	Comments
PARK1	4q21-23	<i>SNCA</i>	α -synuclein	Synaptic protein	LB+	Protein is major component of LB
PARK2	6q25.2-q27	<i>PRKN</i>	Parkin	Ubiquitin-protein ligase	Pleomorphic (most LB-)	Most common cause of AR-JP
PARK3	2q13	<i>SPR?</i>	Aldo-keto reductase?	Unknown	Unknown	Gene not known with certainty
PARK4	4q21-23	<i>SNCA</i>	α -synuclein	Excess of α -synuclein protein		Multiplication of <i>SNCA</i> gene
PARK5	4p14	<i>UCHL1</i>	UCHL1	Hydrolyze small C-terminal adducts of ubiquitin	Unknown	Role uncertain
PARK6	1p36-p35	<i>PINK1</i>	PINK1	Mitochondrial kinase	Unknown	Second most common cause of AR-JP
PARK7	1p36	<i>DJ-1</i>	DJ-1	Oxidative stress protection	Unknown	Rare
PARK8	12p11-q13	<i>LRRK2</i>	LRRK2	Multiple functions by several domains	Pleomorphic (LB+, tau+, ub+)	Most common cause of dominant Parkinson disease
PARK9	1p36	<i>ATP13A2</i>	ATPase type 13A2	Lysosomal protein	Unknown	Complex phenotype (Parkinsonism, spasticity, and dementia)
PARK11	2q37.1	<i>GIGYF2?</i>	GRB10 interacting GYF protein 2	Unknown	Unknown	Role uncertain
PARK13	2p12	<i>OMI/HTRA2</i>	HtrA serine peptidase 2	Serine protease+	Unknown	No cosegregation shown to support pathogenicity
PARK14	22q13.1	<i>PLA2G6</i>	A2 phospholipase	Phospholipid remodelling+	Unknown	Allelic to neuroaxonal dystrophy; adult-onset dystonia-parkinsonism in two patients
PARK15	22q12-q13	<i>FBXO7</i>	F-box protein 7	Phosphorylation-dependent ubiquitination	Unknown	Early-onset, severe phenotype with spasticity and dementia

Table 2: List of most highly implicated PD-linked genes and their association with disease. *LRRK2* protein is encoded by the *LRRK2* gene located in PARK8 locus of Chromosome 12p11.2-q13.1. It is a multifunctional large protein of several domains causing pleomorphic pathology. Mutations in this gene are the most common cause of PD.

1.1.3 Neuropathology

The hallmark of the disease is the degeneration of tyrosine hydroxylase-positive (TH+) dopaminergic neurons in the SN_{pc} of the Ventral Midbrain (VMB), an area of densely packed neurons with melanin pigments, giving it a dark colour (Figure 2) [5, 6]. Loss of these neurons causes dopamine (DA) depletion in the STR, where these dopaminergic axons project (Figure 2) [5, 6]. Symptoms start to appear after ~50% of these neurons have degenerated. In the majority of cases there is aggregation of a pre-synaptic protein, α -synuclein, in inclusions called Lewy bodies (LBs) in the remaining neurons (Figure 3), although the frequency of these inclusions is less in LRRK2-PD than in other forms of the disease [5, 28]. These aggregates are formed due to misfolding of α -synuclein and the build-up of these species is considered by some to be toxic [29, 30]. As the disease progresses, there is additional loss of neurons in other areas of the brain such as ventral tegmental area (VTA) and locus coeruleus and at much later stages, cortex [31, 32]. It has been proposed that PD progresses similar to a prion-like disease, where aggregates are being transported from neuron to neuron via exocytosis-endocytosis, starting from the gastrointestinal system or the olfactory bulbs and then throughout the brain [32].

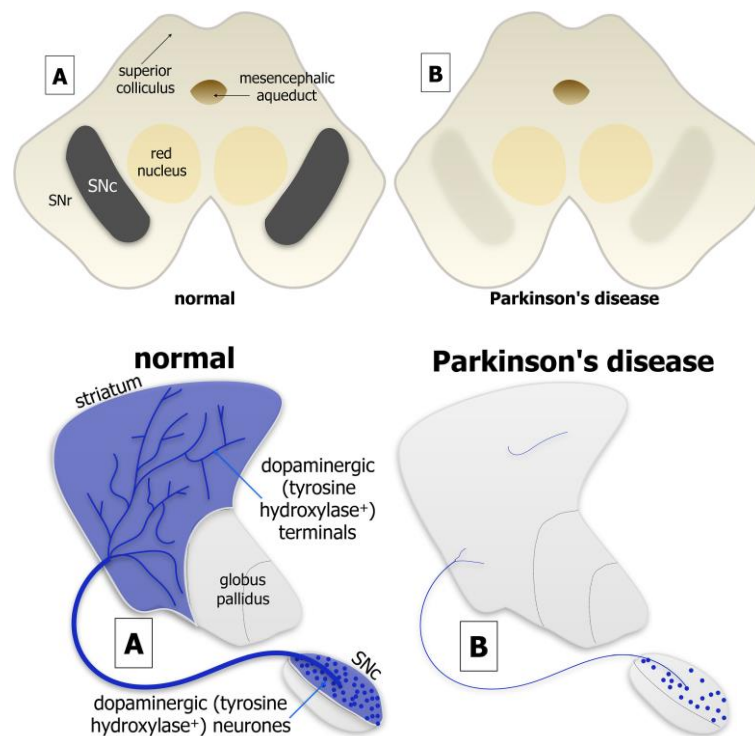


Figure 2: Schematic representation of the VMB and STR in normal (left) and PD brain (right). Top panel showing the loss of melanin pigmented DA neurons in the SN_{pc} of VMB in PD (right) vs normal brain (left). Bottom panel showing the loss of TH neurons in SN_{pc} of VMB causing axonal depletion in STR in PD (left) vs normal brain (right). (Adopted from Run in the Light: Exploring Exercise and Photobiomodulation in Parkinson's Disease).

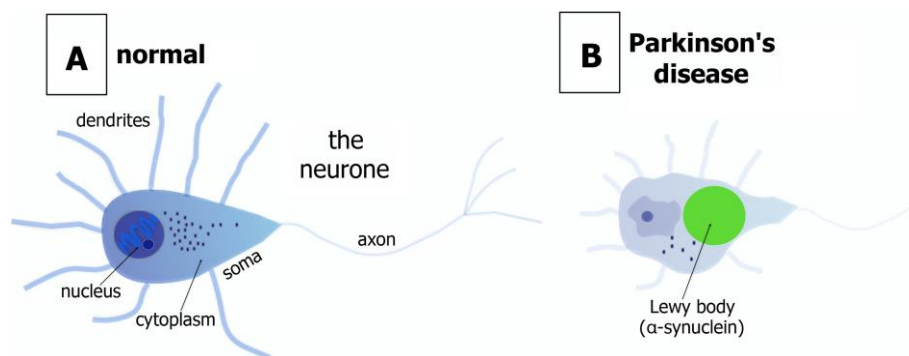


Figure 3: Schematic representation of DA neurons in normal (left) and PD brain (right). DA neurons change morphology and have LBs with α -synuclein aggregates in their cytoplasm in PD (right) vs normal brain (right). (Adopted from Run in the Light: Exploring Exercise and Photobiomodulation in Parkinson's Disease).

1.2 A-synuclein in PD

The physiological role of α -synuclein is not entirely clear, however; multiple lines of evidence suggest a variety of functions in the synapse, including vesicular trafficking, dynamics and maintenance [33-35]. The A53T mutation in the gene coding for α -synuclein (SNCA) located in chromosome 4q21.3, was the first identified to be involved in early-onset PD pathology [16]. Other rare missense mutations have also been reported in the gene (such as A30P, E46K), as well as duplications and triplications of the wild type (WT) locus [17, 36, 37].

A-synuclein is a small, cytosolic protein of 140 amino acids. Under physiological conditions, it is found in a monomeric form, however; it has the tendency to misfold and form oligomers as well as aggregates by yet unclear mechanisms [38]. These higher molecular species are believed to cause toxicity. The majority of α -synuclein found in LBs of PD brains is aggregated and phosphorylated at Ser129, however it is not clear yet whether aberrant increase of this posttranslational modification causes pathogenicity [39]. A schematic of α -synuclein structure is shown in figure 4. It consists of three main regions involving an amphipathic N-terminal domain where most common PD mutations cluster, a “nonbeta amyloid” component (NAC) region responsible for aggregate formation and a C-terminal domain for posttranslational modifications [40, 41].

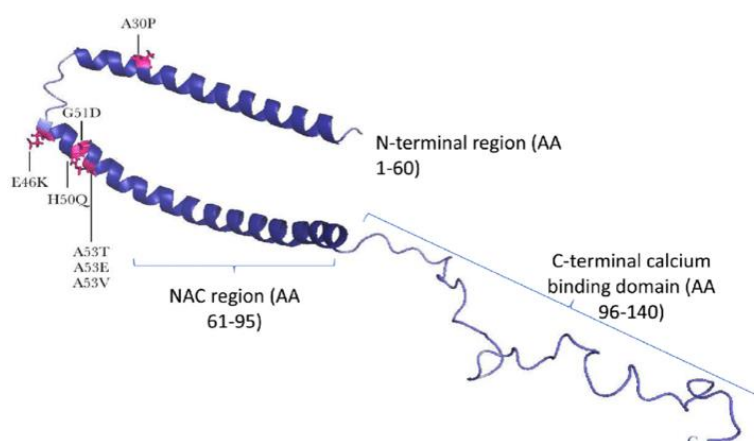


Figure 4: Schematic representation of α -synuclein structure. 14kDa 140 amino acid protein consisting of 3 regions. N-terminal amphipathic domain where PD mutations reside, a NAC region in the middle responsible for aggregate formation and a C-terminal for posttranslational modifications. (Adopted from doi: 10.12688/2Ff1000research.12193.1).

1.3 Mechanisms for PD pathogenesis

PD pathogenesis still needs to be clarified; for most cases, it is suspected that environmental interacting with genetic factors underly the development of the disease. Neuroinflammation due to immune response overactivation, autophago-lysosomal pathway defects, synaptic dysfunction and mitochondrial damage due to reactive oxygen species (ROS) are among the possible reasons that lead to the accumulation of toxic aggregates in LBs and cause neuronal death via autonomous and non-autonomous mechanisms. What follows is a brief summary of the multiple and varying triggers of pathogenesis in PD.

1.3.1 Neuroinflammation

It is not clear yet whether neuroinflammation is the cause or a consequence of neurodegeneration. First evidence of activated microglia in SN_{pc} comes from five post-mortem PD patient brains and this has also been observed in animal models [42]. Microglia cells of the brain's immune system become overactive and produce TNF- α , IL-1 β and IFN- γ proinflammatory cytokines before neuronal loss, indicating that this mechanism precedes neurodegeneration. There is some evidence that early activation of this cell type may help to fight neurodegeneration, however; overactivation enhances the neurodegenerative process. Further stimulation of microglia due to DA neuronal death forms a vicious cycle of neurodegeneration. Astrocytes are also activated further contributing to DA death.

1.3.2 Autophago-lysosomal degradation

Autophagy is a mechanism used by cells to remove cytoplasmic components. There are three major routes involved in this process; macroautophagy for engulfment of large molecules into autophagosomes that fuse with the lysosome, microautophagy for engulfment of smaller molecules via pinocytosis directly by lysosomes and chaperone-mediated autophagy (CMA) where heat shock protein 70 (Hsp70) binds target proteins and sequesters them to the lysosome for binding to lysosome-associated membrane protein 2A (LAMP2A) lysosomal receptor, allowing its translocation inside the organelle. A-synuclein is normally degraded by CMA as described by multiple groups [43-45]. With A53T and A30P mutations there are defects in this pathway with α -synuclein aggregates interacting stronger with LAMP2A,

preventing its internalisation into lysosomes and blocking CMA [45]. Lower levels of LAMP2A and Hsp70 have been reported in SN_{pc} of PD patients associated with downregulated CMA [46]. Additionally, with shRNA-AAV silencing of LAMP2A in the rat STR there is impairment of CMA and accumulation of α -synuclein [47, 48].

1.3.3 Mitochondrial dysfunction and oxidative stress

Neurons require a substantial amount of energy produced in mitochondria, to function correctly and impaired mitochondrial function can lead to neurodegeneration. The first observation that mitochondrial dysfunction is linked to PD was after reports of individuals developing PD-like symptoms as a consequence of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) organic compound exposure [49]. Disrupted complex I of the mitochondrial protein chain has been reported in post-mortem SN_{pc} and STR PD brains [50, 51]. Aggregated α -synuclein has been linked to impaired mitochondrial function and excessive production of ROS by blocking activity of translocase of the outer membrane 20 (TOM20), contributing to neuronal death triggering mechanisms [52, 53].

1.4 LRRK2 in PD

1.4.1 Clinical signs and pathology

The discovery of autosomal dominant mutations in *LRRK2* shed light on the importance that genetics and inheritance play in PD. Mutations in the *LRRK2* gene are the most common cause of familial PD and account for ~10% of such cases and ~1-2% of idiopathic [54-56]. Additional to familial PD, GWAS have shown that variants in *LRRK2* gene can also increase risk for iPD as well as autoimmune diseases (e.g., Chron's disease), highlighting the importance of LRRK2 in the inflammatory response and immunity [26, 27, 57]. Evidence suggests that normal LRRK2 activated by ROS can trigger a pathological cascade in these patients, leading to α -synuclein accumulation and eventually neurodegeneration [58]. LRRK2 patients have been associated with pleomorphic pathology, showing variation in the presence of LBs that accompanies nigral degeneration and often tau-positive aggregation is being reported [18]. Also, presence of LRRK2 in LBs has been contradictory [59, 60]. The diversity observed can, in part, be attributed to the variations in the phenotypes associated with the various mutations in LRRK2 and indicates that pathology can occur even in the absence of α -synuclein contribution, without excluding a potential synergistic effect between the two. In fact, much research has focused on a potential interplay between LRRK2 and α -synuclein; although a direct interaction between the two has not been described, they plausibly act in the same and/or interlinked pathways, which when defective, contribute to PD pathogenesis (discussed in section 1.5). Nevertheless; most LRRK2 patients have a classical PD clinical phenotype indistinguishable from idiopathic patients [61]. In some cases, clinical symptoms of LRRK2 PD are milder, at least regarding non-motor symptoms and lacking a sex difference [62]. Also, cognitive impairment is less common [63]. Age of onset is variable, but most patients develop late-onset PD in their 60s, similar to iPD and are responsive to LEVO-DOPA treatment [64]. PET scans indicate changes similar to typical PD, characterised with reduced ^{18}F -DOPA in nigral DA neurons projecting to the STR [65]. These data suggest common disease mechanisms in LRRK2 mutation carriers and general PD patients, making LRRK2 a promising therapeutic target for different forms of the disease.

1.4.2 LRRK2 structure

LRRK2 is a 2527 amino acid protein encoded in *PARK8* locus, located at chromosome 12p11.2-q13.1, as discovered in 2002, and belongs to the Ras of complex (ROCO) protein family [18, 19, 66]. A schematic of LRRK2 protein structure is shown in figure 5. It is a highly complex structure of several domains with distinct functions, including a catalytic enzymatic core of a serine/threonine (S/T) kinase domain responsible for phosphorylating its target proteins and a Ras-like GTPase (ROC) followed by C-terminal of ROC (COR) GTPase domain for GTP hydrolysis and regulation of dimerisation [67-69]. Additionally, it has non-enzymatic domains of various roles. At the N-terminus it has an armadillo repeat domain (ARM) responsible for structural functions and vesicular dynamics as well as a predicted binding site for various proteins (e.g., Rab10) [70, 71]. ARM is followed by an ankyrin repeat domain (ANK) and a leucine-rich repeat (LRR) domain responsible for protein-protein interactions. Between ANK-LRR there is a cluster of residues (S910/935/S955/S973 etc.) serving as heterophosphorylation sites for other proteins to activate LRRK2 (shown in blue in figure 5) [72-74]. At the C-terminus, WD40 domain acts as a scaffold and facilitator for protein-protein interactions [75-77]. This is also essential for the intrinsic kinase activity, at least *in vitro*, and also in synaptic vesicle trafficking [18, 19, 66, 78, 79]. Hence, LRRK2 can act as a protein scaffold in multiple cellular pathways and confer dual enzymatic function. Additionally, apart from phosphorylating other proteins, LRRK2 has the ability to autophosphorylate itself at various residues mostly found at ROC and kinase domains (shown in red in figure 5) [80]. First *in vitro* studies with mass spectrometry identified T1343, T1491, T1503, T1967 autophosphorylation residues and the discovery of S1292 followed afterwards [80-82]. This latter site has been studied the most due to lack of commercially available antibodies for the rest of these sites.

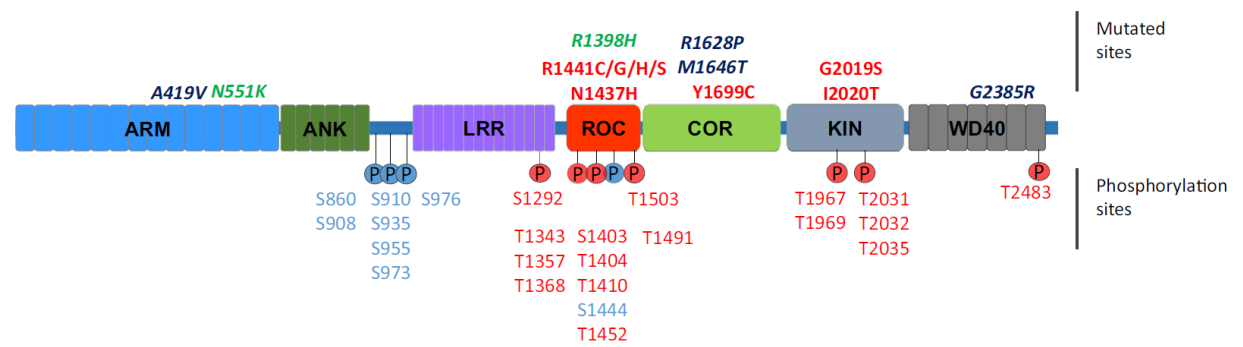


Figure 5: Schematic representation of LRRK2 structure. Multidomain protein consisting of ARM, ANK, LRR, ROC, COR, KIN and WD40 domains. Mutated sites are shown above the schematic including pathogenic mutations clustering in the ROC-COR and KIN domains (red), risk factor mutations (blue) and mutations that confer reduced risk for PD (green). Phosphorylation sites are shown below the schematic including autophosphorylation sites (red) and heterophosphorylation sites (blue). (Adopted from *npj Parkinson's Disease* (2023) 9:104 ; <https://doi.org/10.1038/s41531-023-00544-7>).

1.4.3 *LRRK2* gene mutations

Over 40 variants have been reported in the *LRRK2* gene, of which eight are considered pathogenic (G2019S, R1441C/G/H/S, Y1699C, I2020T, and N1437H), others increase the risk of developing the disease (G2385R, R1628P, A211V, A419V, K544E and M1646T) while one may act protectively (R1398H) [83-87].

- G2019S

The most common mutation found in ~4% of familial and in 1% of idiopathic cases is G2019S, located in the kinase domain of the protein. It was discovered the year following the initial cloning of the gene, in 2005 [54, 56]. There is variation of affected people worldwide across different populations with the highest frequency in patients of North African descent (~37%) and in Ashkenazi Jewish (~23%) families [88, 89]. Remarkably, a very high frequency is also observed in iPD (~41% North Africans and 13% Ashkenazi Jewish) [61]. It is characterised by incomplete penetrance (controversial data, with a range between 24-100%), where it is not yet possible to predict whether carriers will develop the disease or not, which is one of the long-term goals and extensions of this biomarker work. For this reason, it is likely that the identification of G2019S carriers in patients without an evident family history is often misclassified as iPD. Risk in carriers increases with age but is irrelevant to sex, ethnicity or homozygous/heterozygous status (these show similar phenotypes) [83, 89, 90]. The reason for this is unknown, however; there are several polymorphisms in other genes (i.e., *SNCA*, *MAPT*, *CORO1C*) that may act as modifiers of *LRRK2* and trigger disease onset [91-93].

- R1441C/G/H

The second most common mutation in the *LRRK2* gene is R1441C, found mostly in the European and US population [61]. Again, it is characterised by incomplete penetrance and is also found in apparently idiopathic cases. There are several other pathogenic substitutions in this residue, with R1441G found predominantly in the Basques and R1441H/R being less common [19, 61].

- Y1699C and I2020T

Y1699C and I2020T are also pathogenic but less common in the general population [61].

- G2385R and R1628P risk variants

Several studies aimed to explore whether common variants of this gene could represent risk factors for PD. High-throughput GWAS allowing to genotype hundreds of thousands of SNPs and cover the whole genome in patients and controls showed G2385R variant to be present in PD cases much more frequently and the R1628P variant was twice as frequent [85, 94]. Discovering how these variants can increase the risk of PD might provide important insights into understanding the pathogenesis of the disease. In cellular and *in vitro* studies, work from our lab has shown that the G2385R risk variant leads to significantly reduced dimers, yet those dimers exhibit markedly higher intrinsic kinase activity than their WT counterpart [95]. As a large gene, *LRK2* displays a high polymorphic content in terms of single nucleotide substitutions. No deletions or duplications have been identified until now.

1.4.4 LRRK2 enzymatic activity

As mentioned previously, LRRK2 possesses both a kinase and a GTPase signalling domain. It has been consistently seen in experimental models that all pathogenic mutations increase kinase activity leading to neurotoxicity [78, 96-98]. Kinase activity can be assessed directly by measuring autophosphorylation levels of LRRK2 at S1292 residue and/or its *in vitro* intrinsic activity and indirectly by its substrate phosphorylation levels [80, 99]. Apart from the two kinase domain mutations, the majority of pathogenic mutations influencing kinase activity occur in the ROC-COR GTPase domain (figure 5) [100]. As a result, a potential interaction mechanism between the two domains for co-regulation has been extensively studied.

- GTPase activity

LRRK2 function is analogous to small GTPases that alternate between an "active" GTP-bound and an "inactive" GDP-bound state in the presence of GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) and act as molecular switches on their protein targets. However; the requirement for GEFs in LRRK2 GTPase activity is controversial, with some suggesting it is not necessary. Several groups have tested the hypothesis, at least *in vitro*, that GTPase activity regulates kinase activity. There is general agreement that disruption of the GTPase nucleotide binding pocket of LRRK2 by introducing K1347A and T1348N mutations (decreased ability for GTP binding) decreases kinase activity, as measured by S1292 autophosphorylation and *in vitro* kinase activity with myelin basic protein (MBP) as a model substrate [80, 96, 101]. R1441C/G and Y1699C in the ROC-COR domain decrease GTPase activity, although there are mixed results on how these may affect GTP binding [81, 102]. Kinase activity is not required for GTP binding as shown by kinase-dead mutants D1994A and K1906M where LRRK2 was still able to bind GTP in cell lysates [96, 101]. Hence; LRRK2 kinase activity requires GTPase activity, whereas GTPase activity functions independently of kinase activity. Pathogenic mutations in or near the ROC-COR domain shift the balance to the more GTP-bound state (less GTP hydrolysis) and enhance kinase activity, probably via altering GTPase activity. In contrast, the G2019S and I2020T mutations in the kinase domain have no effect on GTP binding and G2385R risk factor has no effect on either kinase or GTPase activity [96]. Kinase activity also decreases by either enhancing or inhibiting GTP hydrolysis [101].

Neurotoxicity has been observed with G2019S, I2020T, R1441C, and Y1699C mutations in embryonic cortical primary neurons and other cell culture models, compared to kinase-dead and GTPase-dead constructs, hence; neuronal death depends largely on kinase activity and indirectly to GTPase activity (probably through altering kinase function) [98, 103]. The R1441G/G2019S double mutant causes neurite outgrowth defects and filamentous distribution of LRRK2 in rat embryonic hippocampal neurons, however; no such change was seen when expressing these mutations alone [80]. Introduction of the kinase-dead D1994A or the S1292A mutated site in the double mutant reversed these effects, suggesting that in addition to kinase activity, S1292 autophosphorylation is required for PD mutations to induce pathogenesis, although this finding awaits replication [10]. On the other hand, pS1292 is not needed to elicit kinase activity, as activity of S1292A and WT LRRK2 was comparable in *in vitro* assays with human embryonic kidney (HEK) 293 cells. With GTPase inactive mutants, there is neurite outgrowth deficit to a similar extent as with G2019S, whereas with a GTPase enhanced mutant there is an intermediate effect. Nguyen *et. al.* showed that genetically enhancing GTP hydrolysis (R1398L) or disrupting GTP-binding (T1348N) is sufficient to protect against G2019S LRRK2 in the rat brain [104].

Collectively, these data suggest a complex regulatory interaction between the two enzymatic functions and that kinase activity is highly dependent on normal GTPase domain function but not *vice versa*. Since GTPase domain regulation is necessary for kinase activity, this could offer an alternative therapeutic strategy for targeting the toxic enhanced kinase activity observed in PD patients, especially by targeting GTP binding capacity of LRRK2.

- Kinase activity

The G2019S LRRK2 mutation has been consistently found to increase kinase activity at least 2-3-fold over WT [78, 105]. There have been discrepancies in the literature regarding the other common pathogenic mutations (R1441C/G/H, Y1699C, I2020T), possibly due to the different models and/or techniques used, however; it is now widely accepted that all pathogenic mutations in both kinase and ROC-COR domains increase kinase activity of LRRK2.

One of the early biochemical assays measuring kinase activity of isolated LRRK2 was carried out by West and colleagues where biotinylated MBP phosphorylation in response to G2019S and R1441C mutants was assessed [105]. They showed that incorporated ³²P in MBP phosphorylation was significantly higher for G2019S mutant compared to WT protein and a non-significant trend for R1441C. When they assessed LRRK2 S1292 autophosphorylation levels by western immunoblotting (WB), there was a significant increase with both mutants, although more robust for G2019S indicating, higher activity [105]. Other assays have been developed since then using different physiological LRRK2 substrates as a phosphorylation readout such as proteins belonging to the ezrin/radixin/moesin (ERM) family and the artificial peptide LRRKtide (RLGRDKYKTLRQIRQ sequence found in all three ERM proteins), resulting in the same outcomes [78, 106]. Furthermore, by comparing various PD mutations with WT LRRK2 in HEK 293 cells, it has been shown that pS1292 levels are increased with G2019S, I2020T, R1441G/C and N1437H and a further synergistic increase is observed when R1441G/G2019S are expressed together, whereas no significant change is observed with Y1699C and G2385R risk factor [80].

Autophosphorylation at S1292 has also been detected *in vivo* in brains of bacterial artificial chromosome (BAC) transgenic mice overexpressing LRRK2. These levels were undetectable in kinase-dead mice, again indicating that kinase activity is necessary for autophosphorylation [80]. In agreement with cell culture studies, overexpressing the G2019S mutation in these mice also causes a robust increase in pS1292 as examined in whole-brain lysates as well as in distinct regions analysed separately (STR, cortex, midbrain, cerebellum, olfactory bulbs) [80]. It is believed that the WD40 domain at the C-terminal is crucial for LRRK2 autophosphorylation as well as for self-interaction [80, 107]. Hence, there is strong evidence that S1292 autophosphorylation consistently increases with G2019S mutation in *in vitro* and *in vivo* models and somehow contributes to PD pathogenesis. However, *in vitro* activity of LRRK2 may not correlate with that *in vivo*.

1.4.5 LRRK2 conformational state

LRRK2 predominantly exists as a dimer (~550 kDa) and at a lower proportion as a monomer (~250 kDa) and within a larger complex (~1.2 MDa), acting as a scaffold for interaction with other proteins [100]. Two independent groups suggested that the kinase active molecule is the dimeric form, as measured by MBP phosphorylation and S1292 autophosphorylation, hence; dimerisation is necessary for this action [100, 108]. However; even though the authors showed that the activity measured was concentrated in fractions the size of a dimer, they did not show that this was indeed dimeric LRRK2 with the method used. Indication that kinase-dead and GTPase-dead constructs prevent dimerisation and favour higher molecular complexes of LRRK2 implies that both enzymatic functions are necessary for the stability of the dimeric molecule [100, 107]. With the G2019S and I2020T mutations in the kinase domain there is a shift to more dimer formation *in vitro*, resulting in an increase in kinase activity and a decrease in GTPase activity [100]. GTP binding but not GTP hydrolysis is necessary for LRRK2 dimerisation, which is in turn is needed for kinase activity, however; both GTP binding and hydrolysis act in an additive manner and are required for normal LRRK2 kinase activation [101, 107, 108]. Additionally, the dimer state is necessary for autophosphorylation even though autophosphorylation occurs in cis (as monomers are not phosphorylated) [107].

1.4.6 LRRK2 signalling pathways

As mentioned previously, due to its multi-domain structure, LRRK2 has the ability to act as a scaffold in various signalling cascades performing its functions with many regulators acting upstream and downstream. Apart from its autophosphorylation ability influencing its kinase activity, it gets phosphorylated by upstream kinases at a cluster of heterophosphorylation sites (S910/935/S955/S973 etc.), located between ANK-LRR domains [97, 109]. This activates LRRK2 to phosphorylate its downstream substrates and cause a cascade of signalling events that influence gene expression and cellular functions. Some of these downstream substrates of LRRK2 may in turn cause its activation acting in a feedback loop.

- Upstream signalling

Since its identification, a series of kinases have been characterised to phosphorylate and activate LRRK2, including TANK-binding kinase 1 (TBK1), protein kinase A (PKA) and casein kinase 1 α (CK1 α) [73, 110]. It is possible that these proteins are themselves regulated by LRRK2 kinase activity. An important feature of LRRK2 activation by these kinases is modulation of LRRK2 binding to 14-3-3 and its isoforms (β , γ , ϵ , η , ζ , and τ/θ), a protein highly expressed in the brain and implicated in PD and other neurological disorders [74, 97, 111]. Within the cell, LRRK2 is mainly cytosolic, but is associated with membranes in a cyclic manner during its activation cycle [112, 113]. Its phosphorylation at S910 and S935 is normally required for binding to 14-3-3 and this interaction impedes LRRK2 dimerisation and its cellular localisation [97]. Decreased phosphorylation of LRRK2 at the S910/935 sites with mutant LRRK2 (R1441C/G/H Y1699C and I2020T), inhibits its interaction with 14-3-3 and causes accumulation of LRRK2 in inclusion bodies in the cytosol [97]. Phosphorylation at S910/935 residues does not directly affect kinase activity but pharmacological inhibition of kinase activity dephosphorylates these residues, at least with some inhibitors, hence; kinase activity indirectly controls 14-3-3 binding and LRRK2 localisation by a yet unknown mechanism involving an upstream kinase [97, 106, 109, 114]. In cultured neurons with overexpression of G2019S or R1441C, there is neurite rescue by inhibiting LRRK2 interaction with 14-3-3 [97, 115, 116]. Interestingly, there is no reduction in the levels of pS935 with G2019S-dead mutants as seen with kinase inhibitor treatment, suggesting an upstream rather than a downstream mechanism

[109, 114]. Thus, inhibition of LRRK2 kinase activity induces a reduction in phosphorylation levels at pS1292 (directly) and at the constitutive sites (indirectly).

Rab GTPases are major endogenous substrates of LRRK2 (the role of Rab GTPases as LRRK2 substrates is discussed in detail in sections 1.6-1.7), however; up to date, Rab29 (also known as Rab7L1 encoded by PD-risk *RAB29*) and Rab12 have been proposed as both upstream and downstream signalling partners of LRRK2 [24, 117-119]. Rab29 activates LRRK2 in cells, recruiting it in a GTP-bound state to TGN possibly via ANK domain interaction. With LRRK2 mutations this TGN translocation is increased, enhancing its activation. Rab12 promotes its localisation to damaged lysosomes where it phosphorylates Rab10, an effect enhanced with R1441C *in vitro* [117]. These Rab10 levels were decreased in Rab12 knockout (K/O) cells and K/O mice [118].

Much less is known for the counteracting mechanisms of LRRK2 phosphorylation. A strong candidate is protein phosphatase 1 (PP1) known to dephosphorylate LRRK2 at S910/935/955/973 [120]. Protein phosphatase-2A (PP2A) is another phosphatase that also dephosphorylates LRRK2 at these residues by interacting with its ROC domain [121]. According to recent evidence, protein phosphatase Mg²⁺/Mn²⁺ Dependent 1H (PPM1H) mediates LRRK2 signalling by dephosphorylating Rab10 [122]. However; neither PP1 nor PP2A cause dephosphorylation of Rab proteins [122].

- Downstream signalling

As LRRK2 is implicated in different cellular functions (discussed in next section), it has various effector proteins that stimulate downstream signalling events. Several members of the GTPase family have been reported as direct endogenous substrates of LRRK2 mainly involved in vesicular trafficking. Additionally, other downstream regulators have been identified, such as NLR family CARD domain-containing protein 4 (NLRC4) and p62 involved in macroautophagy along with LC3 [123, 124].

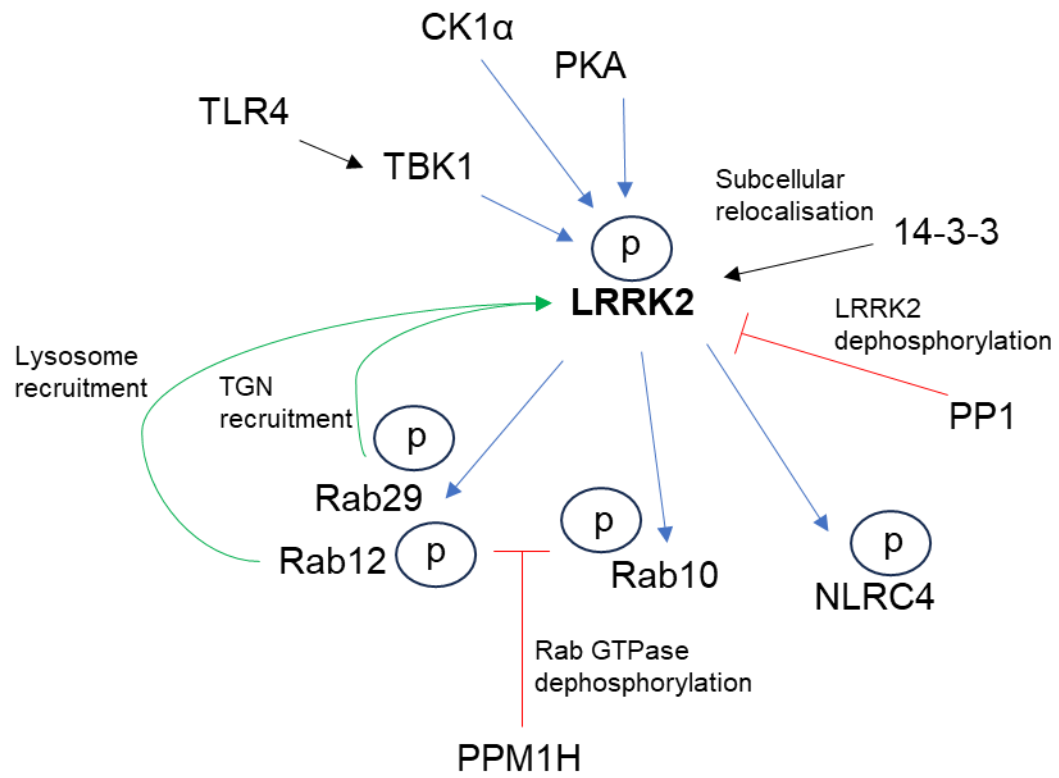


Figure 6: Proposed LRRK2 signalling mechanisms

1.4.7 LRRK2 functions

LRRK2 is expressed in peripheral tissues such as lungs, heart and kidney and in most brain regions in both neuronal and glia cells [59, 125]. The highest expression is observed in STR with lower expression in SN_{pc}, areas highly affected in PD, and the VTA [113, 126-128]. Very high levels have also been reported in immune cells of the CNS (e.g., microglia) and blood (e.g., monocytes, neutrophils, B-cells) [129]. The different functions of LRRK2 are still under investigation, with many of those disrupted in PD.

- Immune response

LRRK2 is very abundant in microglia that become highly activated in PD and it is believed that LRRK2 kinase activity may regulate the inflammatory process [130, 131]. Toll-like receptor (TLR) 2/4/6 and 9 stimulation by lipopolysaccharide (LPS) toxin in macrophages increases pS910 and pS935 levels and leads to proinflammatory cytokine production, a response exacerbated in PD [73]. With LPS injection in the SN_{pc} of mice and activation of the TLR4 proinflammatory pathway, LRRK2 expression is increased in primary microglia and inflammation is attenuated in LRRK2 K/O cells or with kinase inhibition, as shown with TNF- α , IL-1 β cytokine and iNOS reduction. [131, 132]. Inflammation also decreases in LRRK2 K/O macrophages where activation of inflammasomes containing NLRC4 is insufficient [123]. With microglia overexpressing aberrant LRRK2 (G2019S), interferon- γ (IF- γ) stimulation over-activates the nuclear factor of activated T-cell (NFAT) pathway that plays a central role in inflammation and neuronal development, leading to cytokine production [133]. This causes neurite defects and neurotoxicity seen in PD.

In addition to brain cell expression, LRRK2 is also abundant in blood immune cells of the periphery. Increased levels have been reported in CD16+ monocytes, T-cells and B-cells of PD patients and this increase is induced in monocyte cultures upon IFN- γ stimulation [134]. LRRK2 is a negative regulator of T-cell activation in healthy individuals and alterations in LRRK2 function seen in PD may result in poor regulation and result in a pro-inflammatory environment. These findings highlight the importance of inflammatory mechanisms outside the CNS that may contribute to PD acceleration and progression [18, 19]. DA neurons show increased vulnerability to inflammatory insults, hence; in an overactivated microglia environment there may be non-autonomous cell death.

- Vesicular trafficking

Molecules that get internalised within cells by endocytosis are carried by membrane vesicles to various destinations, depending on their cargo. Some molecules are recycled back to the plasma membrane or within the TGN. Those targeted for degradation get fused with early-late endosomes and eventually lysosomes. LRRK2 is greatly involved in the endolysosomal system mainly through its interaction with Rab GTPases, which are key regulators in the endocytic and secretory pathways. LRRK2 pathogenic mutations disrupt Rab activation state and in turn Rab effector proteins causing downstream defects in membrane trafficking often seen in PD. Dysregulation of endosomal dynamics, mediated by LRRK2 mutations, can lead to altered vesicular trafficking and contribute to accumulation of toxic α -synuclein aggregates and PD development. Rab GTPase functions and relationship with LRRK2 is discussed in detail in sections 1.6-1.7.

- Autophagy-lysosomal degradation

Several lines of evidence implicate LRRK2 in autophagy, a mechanism cells use to degrade their components that malfunction in PD [135-137]. G2019S and R1441C LRRK2 mutations promote excessive macroautophagy as seen by increased number of autophagosomes and multivesicular bodies *in vitro* and *in vivo* [138-140]. In these models, there is decreased colocalization of the microtubule-associated proteins 1A/1B light chain 3A (LC3), a marker of autophagosome formation, with the LAMP1 lysosomal marker, indicating defective fusion of autophagosomes with the lysosome and failure of content removal [138-140]. Furthermore, with mutations, LRRK2 interacts excessively with LAMP2A, downregulating CMA [135, 141]. LRRK2 knockdown (K/D) in macrophages and microglia decreases LC3 and in K/O mice it decreases LC3 and p62 protein, suggesting impaired autophagy [142, 143]. As a result, when these mechanisms are defective in PD there is accumulation of proteins such as α -synuclein leading to cellular stress and neurotoxicity and LRRK2 plays a major role in this autophago-lysosomal dysfunction [45, 137].

- Neuronal death

A strong interaction between LRRK2 and FAS-associated death domain protein (FADD) has been reported in cell lines and at endogenous levels in mouse brain extracts [144]. FADD is part of the extrinsic cell death pathway that activates caspase-8 pro-apoptotic factor triggering a signalling cascade of other caspases and eventually cell death in primary cortical neurons [144]. With G2019S, R1441C, Y1699C and I2020T overexpressed mutants there was further activation of this pathway and this effect was reversed by kinase-dead K1906R mutation, strongly suggesting that LRRK2 is an important modulator of cell death via this mechanism [144].

- Mitochondrial function

LRRK2 has been implicated in the regulation of mitochondrial homeostasis and around 10% is associated with mitochondrial membranes [105]. Mutations in LRRK2 have been associated with alterations in mitochondrial function, including changes in mitochondrial morphology, dynamics and function in primary cortical neurons and iPSCs derived from PD patients [145]. Dysfunctional mitochondria can lead to energy deficits and oxidative stress in cells, which are known factors in neurodegenerative diseases. G2019S LRRK2 mutant overexpressed in mice caused mitochondrial deficits that led to DA neuronal loss [139]. Furthermore, increased ROS production has been reported in HEK 293 cells in response to overexpressed LRRK2 and G2019S, effect not seen with kinase-dead mutant [145]. Mitophagy is a process by which damaged or dysfunctional mitochondria are removed and recycled by the cell. Mutant LRRK2 has been linked to impaired mitophagy *in vitro* (R1441C) and *in vivo* (G2019S), which can result in the accumulation of damaged mitochondria and further exacerbate mitochondrial dysfunction [146, 147].

1.5 Evidence of LRRK2 and α -synuclein relationship in aggregation and neuroinflammation

As mentioned before, the majority of LRRK2 PD patients have tau aggregates and ~50% show α -synuclein LB pathology. This implies that there can be PD pathology without aberrant α -synuclein aggregation but the absence of aggregates does not exclude a potential interaction between the two. Experiments have shown that there is a relationship in important cellular functions highly affected in PD such as the immune response, vesicular dynamics, autophagy etc. Several studies have concluded that α -synuclein is not a direct substrate of LRRK2 phosphorylation [148, 149]. Instead, plausible interaction occurs via intermediate common regulators that act in the same pathway or in convergent pathways within the same cell or in neighbouring cells via non-cell autonomous mechanisms. Defective α -synuclein can be secreted from cells by exosomes and stimulate neighbouring cells via cell-cell communication, resulting in toxicity and eventually degeneration [150].

Most *in vitro* and *in vivo* experiments highlight a potential relationship based on inhibition of LRRK2 kinase activity. LRRK2 mutations cause α -synuclein aggregation that is shown to be reversed by kinase inhibition [151]. As mentioned earlier, aggregates of α -synuclein predominantly consist of the phosphorylated form of the protein at S129. Increased levels of pS129 have been reported in rodents overexpressing G2019S LRRK2, suggesting a possible acceleration of aggregation with this mutant form of LRRK2 [152, 153]. This effect was effectively reversed in primary cortical neurons via kinase inhibition, highlighting the importance of kinase activity of LRRK2 in the aggregation process [154]. The group of Lin *et. al.* also investigated a potential synergistic role of both proteins in PD pathogenesis using transgenic mice. It was shown that by combining the overexpression of WT or G2019S LRRK2 with A53T α -synuclein double transgenic mice worsen neurodegeneration seen with either transgene alone [148]. LRRK2 promoted α -synuclein aggregation that was delayed in A53T mice with LRRK2 K/O, suggesting that LRRK2 enhances toxicity in α -synuclein pathology [148].

A clear relationship between LRRK2 and α -synuclein was observed in Schapansky *et. al.*, where K/I of G2019S mutation in mice caused lysosomal dysfunction that resulted in aberrant α -synuclein clearance and accumulation as well as increased α -synuclein release [137]. Daher and collaborators showed that LRRK2 K/O rats are protected against LPS administration or recombinant adeno-associated-virus (rAAV)- α -synuclein injection in the SN_{pc} [155]. The same group tested later whether pharmacological LRRK2 inhibition could prevent dopaminergic

neurodegeneration in these mice, and indeed, after treating G2019S LRRK2 transgenic rats overexpressing rAAV- α -synuclein with a potent and selective LRRK2 kinase inhibitor (PF-06447475), there was blockage of α -synuclein induced neurodegeneration, assessed by TH cell preservation in the SN_{pc} and STR [151, 156]. In addition, in the treated group there was reduced neuroinflammation associated with G2019S LRRK2 expression, concluding that G2019S LRRK2 expression augments α -synuclein induced dopaminergic neurodegeneration [151]. Exacerbated α -synuclein accumulation and neurodegeneration has also been reported in transgenic mice overexpressing G2019S LRRK2, accompanied by deficits in motor abilities [157]. Further evidence that the two proteins interact comes from LRRK2 K/O primary microglia cells, where with LPS or α -synuclein fibril treatment there was a reduction in the levels of pro-inflammatory cytokines [131]. This reduction is likely mediated through the loss of kinase activity of LRRK2, as after LPS exposure together with GSK2578215A inhibitor treatment, the inflammatory response was also attenuated [131]. However; contradictory findings in a different study show that pre-formed fibrils injected in the STR of mice followed by MLI-2 LRRK2 kinase activity inhibition, had no effect on rescuing neurodegeneration or motor deficit improvement, suggesting that LRRK2 is not involved in the pathology caused by α -synuclein [158]. These discrepancies in results likely arise from the different models and techniques used, nevertheless; the link (or lack of) between these two proteins remains an area of active investigation as it pertains to PD pathogenesis.

Implication of LRRK2 in iPD and α -synuclein pathology was examined in a proximity ligation assay experiment in post-mortem iPD brains as well as in two rat models overexpressing α -synuclein via AAVs [58]. It was shown that in iPD, normal LRRK2, irrespective of mutations, becomes activated in dopaminergic neurons in response to ROS and causes an increase in Rab10 and S1292 phosphorylation as well as reduction in 14-3-3 in the SN_{pc}. This ultimately led to α -synuclein accumulation due to defects in the endo-lysosomal degradation system and neuronal death.

Above findings strongly suggest that the two proteins act in the same pathway or in different pathways that “cross-talk”. The effects of LRRK2 on α -synuclein are more likely indirect; an upstream effect is mediated by implication of LRRK2 in autophago-lysosomal dysfunction resulting in α -synuclein accumulation and a downstream effect is seen in LRRK2 activation in myeloid or microglia cells in response to α -synuclein overexpression [131, 137, 155]. As LRRK2 inhibition prevents α -synuclein aggregation, it may have neuroprotective effects in PD-related LB pathology. Additionally, a potential implication of LRRK2 in iPD can have advantages in

developing drugs to target a broader PD population and not only those affected with LRRK2 mutations.

1.6 The discovery of Rab GTPases as LRRK2 substrates

There are more than 70 members of Rab GTPases identified in humans [159]. Up to date, a subset of 10 different Rab proteins has been discovered as endogenous physiological LRRK2 substrates, including Rab3a/b/c/d, Rab8a/b, Rab10, Rab12, Rab35 and Rab43 [23, 99, 160, 161]. These proteins are mainly involved in vesicular cargo transport within cells by associating with membranes and act as molecular switches [99]. Once synthesised, Rab proteins are inserted into membranes to ensure precise delivery of cargos to their intended destinations. They exist in a cyclical GDP/GTP bound state controlled by GAPs, GEF and GDP-dissociating inhibitors (GDIs). When GDP-bound, Rabs are inactive and GEFs switch on transport by dissociating GDP to GTP enabling them to associate with vesicles for their transport to target membranes. They then undergo GTP hydrolysis and become GDP-bound again by GAPs to switch off transport. GDIs keep them inactive in the cytosol until recycled to the donor membrane. It is now known that apart from this route, they are also modified and controlled by phosphorylation [160]. Recently, it was discovered that LRRK2 phosphorylates them via its N-terminal ARM domain in their switch II region on S/T residues *in vitro* and *in vivo* under physiological conditions causing a conformational change between GDP/GTP bound state [71, 99, 162]. According to Liu *et. al.*, Rabs need to be GTP-bound and membrane associated in order to get phosphorylated by LRRK2 [23]. This phosphorylation has mostly an inhibitory effect on Rab interaction with their target proteins. [99]. Mutations in LRRK2, regardless of the domain, increase Rab8/10/12 and Rab29 phosphorylation *in vitro* and this aberrant state can have downstream effects on vesicular trafficking, at least in mice [23, 99]. Thus; LRRK2 controls the GTP/GDP-bound state of Rab GTPases and regulates their specific insertion in target membranes, altering their membrane-cytosol equilibrium. It is believed that this happens because hyperphosphorylation favours the GTP bound state, inhibiting GTP hydrolysis [160]. The signal is counteracted by PPM1H phosphatase that dephosphorylates Rab proteins [122].

1.7 Functional roles of Rab GTPases

Different Rab GTPases can bind and act as regulators on various effector proteins when phosphorylated by LRRK2 to influence vesicular trafficking necessary in various cellular functions.

Rab10 and Rab8 are involved in ciliogenesis, a process important in cell stability and developmental signalling [163]. Under normal conditions, Rab10 suppresses cilia formation by inhibiting Rab interacting lysosomal protein-like 1 (RILPL1) effector, while Rab8a has the opposite effect promoting this process [163]. Aberrant Rab10 phosphorylation by LRRK2 prevents cilia formation by further activating RILPL1, as seen in G2019S-human derived iPSCs and in STR neurons of transgenic mice overexpressing R1441C LRRK2 mutant [163]. LRRK2 can also cause centrosomal defects via aberrant phosphorylated Rab8a accumulation in skin fibroblasts and lymphoblasts of G2019S PD patients, an effect that is reversed with kinase inhibition [164]. The centrosome is important in cell polarity and cell cycle division events as well as in microtubule dynamics, which are critical in neurons. Additionally, Rab8a mediates endo-lysosomal trafficking, that gets inhibited with G2019S in cell cultures causing defects in the pathway [165].

As stated previously, Rab29 acts both as an upstream and downstream regulator of LRRK2. The Rab29-LRRK2 interaction is critical in the endo-lysosomal pathway as blocking this complex attenuates LRRK2 recruitment to lysosomes for content release, leading to dysfunction and neurotoxicity [166]. When overexpressed in HEK 293 cells, it increases LRRK2 kinase activity, as measured by enhanced S1292 and Rab10 phosphorylation, which is then abolished with D2017A kinase-dead LRRK2 mutant [24]. This increase was much greater with R1441C/G/H and Y1699C GTP-binding mutations as well as G2019S [23, 24]. Rab29 is localised to the TGN where it recruits LRRK2 for activation, possibly via its ANK domain [23, 24]. With G2019S, R1441C/G and Y1699C mutations, this recruitment is enhanced, further overstimulating LRRK2 [23, 24]. Additionally, with Rab29 K/O and abolishment of interaction with LRRK2 there is a decrease in heterophosphorylation at S910/935/955 and S973 that is indirectly dependent on kinase activity, as stated earlier [24]. As a downstream effector of LRRK2, Rab29 when phosphorylated can no longer activate LRRK2, suggesting a negative feedback loop [24]. There is contradictory evidence, however, in other reports where Rab29 K/O has no effect on downstream Rab phosphorylation *in vitro* and *in vivo*, Rab29 overexpression does not increase LRRK2 kinase activity and Rab29 is not required for LRRK2 recruitment to TGN or lysosome or its activation [167, 168]. Further research is needed to clarify these observations.

Rab12 is also an endogenous substrate of LRRK2, normally involved in endo-lysosomal trafficking. There is considerable evidence now that Rab12, also acts as an activator of LRRK2 by binding to its ARM domain, in a distinct manner to Rab29 [118, 169]. It is believed that it recruits LRRK2 to damaged lysosomes, activating it as seen by increased Rab10 phosphorylation levels, an effect that was enhanced with G2019S and R1441C mutations [118, 169].

Multiple lines of evidence suggest that Rab dysregulation due to LRRK2 mutations can cause neurodegeneration. When transfecting primary cortical neurons with Rab1a/3c/35 phosphomutants abolishing their ability to interact with LRRK2, a shortening of neurites and cell death is observed, with Rab35 causing the most severe damage [160]. Furthermore, by introducing mutant Rab35 using rAAV injections in the SN_{pc} of mice brains, there is significant DA neuronal loss in this specific region [160]. Additionally, by analysing various brain regions of G2019S and R1441C transgenic mice an increase of Rab35 expression is seen in SN_{pc} [170].

Collectively, the above findings highlight the importance of LRRK2 interaction with Rab GTPases in various pathways for cell survival. By overactivated mutant LRRK2 there is hyperphosphorylation of Rabs causing defects in downstream signalling. As a result, the equilibrium of Rab GTPases is disrupted, resulting in neuronal instability and the subsequent DA loss seen in PD. Moreover, overexpression of Rab8a, Rab1, and Rab3A has been linked to α -synuclein toxicity in cellular and animal models of PD, suggesting a functional interplay [35, 171].

1.8 Biomarkers

1.8.1 General

According to Biomarkers Definitions Working Groups *“Biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”* [172]. As PD is a multifactorial disease there is likely a need for several types of PD biomarkers not only for diagnosis at early-stages and monitoring but also to prevent disease before its onset in high-risk populations, as by the time of clinical diagnosis a great number of neurons have already degenerated. Despite progress that has been made in understanding the molecular mechanisms underlying PD, biomarker availability is limited and there is a great need for discovering new approaches. In general terms, there are four main biomarker categories including clinical, imaging, genetic and biochemical factors.

- Clinical

Clinical signs involve motor and non-motor defects as described previously. Clinical evaluation occurs after disease onset and many of the symptoms are not exclusive to PD. Tremor, sleep disorders, depression, cognitive impairment etc., are present in other diseases as well, making accurate diagnosis difficult. As a result, a combination of different biomarkers is necessary.

- Imaging

Positron emission tomography (PET), single photon emission tomography (SPECT), magnetic resonance imaging (MRI) and transcranial sonography (TCS), are among the techniques currently used to assist clinical diagnosis. These mostly rely on structural changes that may occur in PD and on activity of specific brain regions affected using specific radiotracers.

- Genetic

GWAS have identified several candidate genes and SNPs as risk factors for developing PD such as *LRRK2*, *SNCA*, *RAB29*, *GBA* etc. Development of disease is highly variable depending on penetrance of corresponding gene as well as contribution of environmental and lifestyle factors.

- Biochemical

Interest for developing such markers lies mostly in studying the expression levels and activity of proteins associated with PD with many candidates being under evaluation, e.g., LRRK2, α -synuclein, tau and DJ-1. Different biofluids such as blood, urine and cerebrospinal fluid (CSF) are commonly used and are generally considered as non-, or minimally-invasive sources of study. Isolation of blood-specific cell types as well as EVs of ~125nm coming from the periphery are the most common study systems.

1.8.2 LRRK2 and Rab GTPases as PD biomarkers

Most markers based on LRRK2 and Rab GTPases have been successful for target engagement and inhibitor efficacy rather than for the disease itself (prognosis or diagnosis), thus; more research is necessary towards this direction. Focus of this work relies mostly on biochemical biomarkers based on changes in these proteins in blood, urine and CSF biofluids from patients of different genetic backgrounds and in relation to their clinical assessment.

- Blood specific cell types

LRRK2 and Rab GTPases are abundant in blood, mostly in immune cells with the highest levels being expressed in neutrophils [129, 134]. Mixed peripheral blood mononuclear cells (PBMCs) also have very high levels, specifically the monocyte sub-population, whereas B-cells, T-cells and natural killer (NK) cells are characterised by lower levels. Hence; blood is a very rich and easily accessible source to study LRRK2 and Rab GTPases.

It is known from cellular and animal models that different LRRK2 mutations can alter its phosphorylation status. As a result, many groups have recruited patients from different PD genotype backgrounds in order to test whether these changes are also seen in human samples and find potential differences among them. Dzamko *et. al.* isolated PBMCs from blood of iPD patients in order to measure total LRRK2 levels and its phosphorylation at S935 and S910 heterophosphorylation sites and assess whether these could be utilised as biomarkers [173]. There was correlation with total LRRK2 and S910/S935, however; no changes were observed between the groups and there was no correlation with disease duration, severity or medication. In contrast to this finding, an increase of pS935 has been seen in PBMCs from iPD patients by using quantitative ELISA instead of WB; a discrepancy that could be explained by the sensitivity and specificity of the different techniques used for analysis [174] (and our unpublished data). Levels of pS935 are decreased in G2019S carriers manifesting PD compared to iPD or non-manifesting carriers whereas total levels of LRRK2 remain unchanged [174, 175]. In cellular and animal models, G2019S also reduces pS935 to some extent, but has a milder effect than the R1441C mutation, with some reports suggesting that it has no effect at all, hence; these findings imply that distinct mechanisms regulate this post-translational modification (PTM) in human cells and also between the different PD genotypes (iPD compared to G2019S PD but also between G2019S affected and non-affected carriers) [176]. G2019S patients and healthy carriers of this mutation have also shown higher intrinsic kinase activity in

cellular and animal models, but this was only evident in males when both sexes were examined separately indicating a potential sex difference that needs to be further explored in larger samples [174]. The G2019S variant occurs at a highly conserved residue within the kinase domain, leading to specific conformational changes, mediating its increased activity. Although there is conflict of whether LRRK2 heterophosphorylation sites, such as S935, can be utilised as PD biomarkers, they can be used effectively as pharmacodynamic measures of kinase inhibitors in clinical trials (i.e. PF06447475, GSK2578215A), as there is a dose-dependent reduction in activity seen in the different models [173, 177, 178]. Reduction of this phosphorylation is similar among the groups and total levels remain the same except after long exposures (24h). It is contradictory though that this phosphorylation site is decreased in G2019S-PD carriers but also by the use of kinase inhibitors. Additionally, in cells with a kinase dead mutant expression, its levels are as with WT LRRK2.

Although pS935 is appropriate for measuring pharmacodynamic efficacy, it is not a reliable marker of kinase activity, in contrast to the S1292 autophosphorylation site. However; it is hard to detect S1292 in biosamples due to its low stoichiometry and limitations in commercially available antibodies. From the time that Rab GTPases were discovered as major physiological LRRK2 substrates, several studies started focusing on how their phosphorylation levels may be used as a measure of kinase activity [99]. All pathogenic mutations increase Rab10 phosphorylation levels in *in vitro* and *in vivo* models, although to differing degrees, and are decreased with kinase inhibitor treatment [99, 161]. This has been tested in human PBMCs and neutrophils of PD patients. The ratio of pT73-Rab10 and pS935 to total Rab10 and LRRK2 respectively was unchanged in both cell types but phosphorylated levels were reduced to a similar extent with MLI-2 *ex vivo* treatment [179]. As a result, Rab10 is a promising biomarker for target engagement, however; none of these measurements is a direct way to monitor LRRK2 activity as phosphorylation is mediated by other regulators as well, and also counteracted by activity of specific phosphatases (e.g., PPM1H). The only correlation noted is between Rab10 phosphorylation levels and disease severity assessed by Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) in PBMCs [179]. In a different study with few participants, the authors failed to detect any differences between G2019S PD cohort, iPD and healthy volunteers [129]. Total LRRK2 levels were higher in PBMCs than in neutrophils from the same donors whereas S935 phosphorylation was similar. Total Rab10 levels were also similar but phosphorylation was greater in neutrophils, possibly indicating higher activity in this cell type [129]. Additionally, they observed that with PF-06447475 treatment, it takes longer for S935 dephosphorylation to take place than for Rab10,

likely reflecting the intermediary action of other kinases acting on this Ser residue. In a later study, the same group showed that R1441G but not G2019S affected or non-affected carriers caused a significant Rab10 phosphorylation increase in neutrophils, with both WB and MS analysis [180]. However; post-mortem brain lysates from PD individuals carrying G2019S and R1441H mutations as well as iPD were proven unsuitable to detect such changes with highly variable results, at least in cortex tissue [180]. In contrast, Di Maio *et. al.* showed increased pT73-Rab10 phosphorylation levels in SN_{pc} of iPD patients, using a different technique to label phosphorylated Rab10 [58]. They also found a small decrease in S935 of R1441G compared to iPD but only a trend compared to controls and no difference compared to G2019S. This decrease with R1441G is reported in other models as well, as mentioned before. Analysis of PBMC lysates from G2019S PD patients with an electrochemical Meso Scale Discovery-based assay showed increased pT73-Rab10 levels versus iPD and between G2019S healthy carriers versus non-carriers, however; this was after normalising against total LRRK2 and not total Rab10 [181]. The N551K/R1398H LRRK2 protective variant, causes a reduction in Rab10 phosphorylation compared to non-carriers; again, when normalised to total LRRK2, highlighting the importance of this substrate in PD [181]. In this study, the comparisons were made in cellular experimental models, rather than clinical samples, and it was hypothesized that increased LRRK2 GTPase activity underlies this result.

Rab29 is also a biomarker candidate of great importance, as it acts both as a LRRK2 substrate and an activator [24]. It is evident that when Rab29 is unable to bind GTP, this decreases autophosphorylation of LRRK2 when localized at the TGN. According to Purlyte *et. al.*, GTP binding of ROC domain promotes Rab29 activation of LRRK2, thus; as kinase activity has been linked to toxicity seen with mutations, blocking Rab29 could be a potential target for PD therapeutics. However; no current studies are available regarding its expression in blood cells.

- Urine EVs as biomarker tool

Urine can be collected in large quantities and is considered a non-invasive method. Most studies have focused on isolating EVs from urine via various techniques, as LRRK2 is highly abundant in these vesicles [182-187]. West and colleagues measured total and phosphorylated LRRK2 in EVs from iPD and found increased pS1292 levels compared to controls, which correlated with disease severity and were more abundant in males compared to females [183]. Total LRRK2 levels have also been reported elsewhere to be higher in males regardless of disease status [188]. Importantly, pS1292 levels could predict PD risk among G2019S participants as affected carriers had higher levels versus non-affected carriers [183, 184]. Levels of pT73-Rab10 were higher in urine EVs of iPD male patients but not G2019S affected carriers compared to controls and this ratio increased with time. However; there was no correlation between LRRK2 levels [181].

PS1292-LRRK2 could not be detected in urine EVs isolated from macaques, probably due to lower expression levels [186]. In addition to pS935 levels, total LRRK2 also decreased with PFE-360 or MLI-2 inhibition, and this may be explained by the fact that LRRK2 kinase inhibitors reduce LRRK2 exosomal release [182, 186]. In contrast, pT73-Rab10 levels could be more reliably used, as reduction occurred when normalised to total Rab10 levels.

Unfortunately, most studies rely on semi-quantitative techniques such as WB. No current studies have successfully measured LRRK2 or Rab GTPases from urine EVs using a more sensitive approach; however, other PD-related proteins such as total and oligomeric α -synuclein have been successfully measured in ELISA-based techniques and future studies quantifying LRRK2 as well as Rab GTPase substrates using more quantitative methods are necessary [189].

- Neuronal EVs as biomarker tool

As it is possible to study brain tissue from PD patients only post-mortem, alternative strategies of studying the CNS environment have to be found. One such approach is the isolation of EVs from CSF. These have generally much higher pS1292 levels compared to urine vesicles, but there is no difference in these levels between LRRK2 mutation carriers [184]. Total levels of LRRK2 do not correlate between the two biofluids, implying distinct regulatory mechanisms. However; it was possible to measure LRRK2 in a small amount of whole CSF without the EV-enrichment step, as shown in a recent quantitative study where LRRK2 levels are higher in G2019S subjects with PD compared to G2019S non-manifesting carriers, iPD and HC when measured by Stable Isotope Standard Capture by Anti-peptide Antibody (SISCAPA) [187]. Additionally, they showed that LRRK2 levels are higher in older participants, at least in the HC and G2019-PD.

Another way to recover brain EVs is to isolate them from plasma. It is thought that these vesicles are released from the brain to the periphery, transporting their cargo with them; and as a result, they represent a snapshot of the brain's environment at that time. Most studies have focused on AD and other proteins such as tau and α -synuclein. By using an antibody against L1CAM, a neuronal marker for EVs, several groups have managed to isolate brain specific EVs by IP and measure protein levels such as α -synuclein, tau, etc. in their lysate [190-193]. No current studies have been carried out regarding LRRK2 and its expression in these specific vesicles, which is one of this report's interest.

The original cellular source of urine and plasma vesicles is hard to be determined. It is thought that these come from kidney and brain respectively; although there are multiple cell types in these organs from which EVs are released. The above findings highlight the importance of having multiple measurements, as many markers are not detectable in some biofluids/tissues, or various other factors may play a role such as the presence and activity of relevant phosphatases, LRRK2 sub-cellular localisation, upstream regulators activity, cell type, etc.

AIMS OF STUDY

The main objective of this work was to assess LRRK2 changes in multiple biofluid samples among different PD cohorts. There are currently no ways to biochemically discriminate between LRRK2 PD and iPD, as both are characterised by late-onset and similar clinical assessment. Additionally, LRRK2 mutations are characterised by incomplete penetrance, highlighting the importance of finding biomarkers for PD risk prediction in carriers. There is evidence that S1292-LRRK2 autophosphorylation levels are elevated in iPD and can also discriminate between G2019S affected and non-affected carriers, at least in urine EVs. This indicates that the intrinsic kinase activity of the LRRK2 protein is also affected, in an as yet unknown way, in this patient group, however; it has not been assessed yet. Additionally, LRRK2 PD patients commonly develop α -synuclein aggregates in LBs, as seen in post-mortem brain tissues. *In vitro* and *in vivo* models support the notion that LRRK2 and α -synuclein act in common pathways important for cell survival. Their relationship has been extensively examined in eliciting an immune response, where in α -synuclein overexpression models, pathology can be rescued to some extent by inhibiting LRRK2 kinase activity and in LRRK2 deficient rats, viral overexpression of α -synuclein fails to elicit neuronal loss.

Based on the evidence assembled to date, in this study, potential differences in LRRK2 levels, activity and levels of its substrates were examined in G2019S affected and non-affected carriers for disease status prediction. Due to the strong indication that LRRK2 activity is involved in α -synuclein pathology, A53T PD patients were also recruited and this is the first study to evaluate LRRK2 levels in this PD subgroup. The main focus was on blood immune cells as strong evidence supports the hypothesis that the involvement of LRRK2 in α -synuclein induced neurodegeneration, at least in part, is mediated through the immune system. By interrogating LRRK2 status in peripheral immune cells, such as PBMCs and monocytes, in carriers of specific mutations may reveal a unique profile of LRRK2 activation status within this particular patient population. Specifically, total LRRK2 levels, its phosphorylation at S935 residue and *in vitro* kinase activity in PBMCs and CD14⁺ monocytes were monitored. These immune cell types were chosen due to high levels of LRRK2 expression and their implication in the inflammatory process often seen in PD. Various groups have been unable to reliably detect pS1292-LRRK2 signal in PBMCs, possibly due to its low stoichiometry, hence; measuring the *in vitro* kinase activity of LRRK2 could provide an alternative direct way of assessment. The reason for this is not clear, as a robust pS1292 signal has been detected in LRRK2 purified from urinary EVs or EVs isolated

from CSF, however; a Signalbooster was used as the antibody diluent. It is not known yet if phosphorylation at this site is detectable in LRRK2 purified from specific monocyte subtypes, which was examined here. Since LRRK2 has been reported in CSF-derived EVs, it is expected that LRRK2 purified from neuronal EVs present in plasma will also show evidence of phosphorylation at the S1292 residue. For this reason, EVs of neuronal origin from plasma were isolated in an effort to depict potential LRRK2 changes in the brain. These EVs represent a snapshot of the brain's environment that is otherwise difficult to be studied. Additionally, phosphorylation of several Rab GTPases may serve as a pharmacodynamic measure and an indirect indication of LRRK2 activity as seen before with Rab10 in PBMCs of iPD and LRRK2 patients, however; not yet studied in A53T-PD. The expression pattern of Rab29, which acts both as a substrate and an activator of LRRK2, has not been examined in immune cells before. Additionally, whether these LRRK2 substrates are present in EVs remains to be elucidated. As a result, their expression and potential differences according to different PD status was investigated here. As PD is a progressive disorder, monitoring protein changes through time is important, hence; this study consisted of two visits of biofluid collection, 6-months apart, regarding one of the two cohorts analysed.

The final part of this project was to produce an ultra-sensitive multiplexed LRRK2-pathway focused ELISA-based array. There is a lack of high-throughput assays and most studies focus on semi-quantitative techniques for analysis. The advantage of this method is that it is capable of quantifying all of these pharmacodynamic markers of LRRK2 simultaneously. It involves capture antibodies, micro-printed on clear bottom wells as different "spots" and uses a special microplate reader equipped with optics to image the well and quantify the signal from each "spot". In this way, it is possible to obtain total and phosphorylated LRRK2 signal from the same sample, at the same time, minimising normalisation errors. The goal was to set up the assay to measure total and S935 phosphorylated levels of LRR2 in CSF and immune cells in a small cohort. In this way, this assay can be utilised later in larger scale studies, where additional "spots" of other antibodies of the LRRK2-signalling pathway could be included, i.e., Rab GTPases.

Overall, this work will provide new insights regarding A53T pathology and LRRK2 activity in various human biofluids and direct comparison between iPD and G2019S related PD. In this way, distinct mechanisms of LRRK2 may be revealed in the different cellular systems of the same individuals in different forms of PD and such changes may correlate with disease prediction, severity and progression over time. There is a critical, unmet need for the development of reliable, specific, and sensitive biomarkers, not only of disease progression, but also as markers of pharmacodynamic

measurements in future clinical trials of disease-modifying compounds. Ideally such a marker would be one that is generally altered in most forms of PD, and one for which changes are evident early on in the course of the disease or prior to symptom onset for those at-risk populations.

MATERIALS & METHODS

3.1 Study participants/Biofluid collection

Demographics and key clinical features of the two cohorts are summarized in Tables 3-5. All participants gave consent for participating in this study over two clinical visits (six months apart). For the Athens cohort, biofluids assessed (blood, urine, CSF) were collected at the Departments of Neurology at Henry Dynan Hospital Center (Athens, Greece) and Aiginiteio University Hospital (Athens, Greece). For the Columbia University Medical Centre (CUMC) cohort, biofluids were collected at Columbia University (New York, USA) and shipped for analysis. Samples were coded at the time of collection and the patient/sample data stored at the clinic, so that no identifying information concerning the sample was available to the researchers performing the isolations and assays. Motor and non-motor assessment was performed using the modified MDS-UPDRS (4 modules) and cognitive performance was scored using the Montreal Cognitive Assessment (MoCA). This study was approved by the Ethics Committee of the Biomedical Research Foundation Academy of Athens. Exclusion criteria for patient groups include a diagnosis for other neurodegenerative diseases, or specific signs of disorders such as progressive supranuclear palsy, multiple system atrophy, Huntington's disease, corticobasal degeneration, or neurologically involved autoimmune disorders. Inclusion criteria for the HC group includes lack of recent medical history of urinary, bladder, or other infections requiring the treatment with antibiotics, an absence of a diagnosis of PD in a first-degree blood relative, or other neurodegenerative or autoimmune disease.

3.2 Isolation of PBMCs

Blood was collected in Heparin-coated Vacutainer collection tubes (BD) and 15 ml were mixed with an equal amount of 1X Phosphate Buffered Saline (PBS) (Gibco). Eight ml blood was slowly layered on top of 4 ml Lymphosep (Biowest) in 15 ml falcon tubes. Centrifugation for 30 min, at 800 g, at 20 °C followed. After centrifugation, the band of PBMCs was carefully collected (~2 ml) in falcon tubes and 5 ml of 1X PBS was added. Centrifugation for 15 min, at 1,500 rpm, at room temperature (RT) followed. The supernatant was discarded and pellets were resuspended in 1 ml 1X PBS and transferred to 1.5 ml eppendorfs. A spin for 5 min, at 2,000 rpm, at 4 °C followed, supernatant (S/N) was discarded and pellets were stored at -80 °C. Some cells were treated for 2h with dimethyl sulfoxide (DMSO) (Sigma) or MLI-2 (Merck) at 200-500 nM, a potent and selective LRRK2 inhibitor in Roswell Park Memorial Institute (RPMI-1640, Sigma) medium, then collected/washed in 1X PBS and stored as a pellet at -80 °C. Prior to biochemical analysis, pellets were lysed with 50 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM ethylene glycol tetraacetic acid (EGTA), 270 mM Sucrose, 1% Triton + 1X protease ethylenediaminetetraacetic acid (EDTA)-free/phosphatase cocktail inhibitors (Roche) added fresh), for 30 min on ice. Lysates were then centrifuged at 45,000 g, for 1 h, at 4 °C and S/N was collected and stored at -80 °C.

3.3 Isolation of monocytes (CD14⁺)

Blood was collected in K₂-EDTA Vacutainer collection tubes (BD), and 15 ml of diluted blood (in PBS) was transferred to a 50 ml centrifuge tube. In the diluted blood, 750 µl of "Isolation Cocktail" from the EasySep™ Direct Human Monocyte Isolation Kit (StemCell Technologies) were added. "RapidSphere Magnetic Beads" from kit were vortexed for 30 sec before use and added to the blood sample. The blood was mixed gently by tube inversion and incubated for 5 min, at RT. The tube was topped up to 50 ml with 1 mM EDTA-PBS Ca²⁺/Mg²⁺ free (Gibco), the solution was gently mixed and falcon was placed into a magnet (StemCell Technologies) without the lid, for 10 min. The enriched monocyte cell suspension was carefully removed (leaving ~10 ml behind) into a new 50 ml falcon, 750 µl of beads were added and tube was gently mixed followed by 5 min RT incubation. Cell suspension was again removed (leaving ~5 ml behind) and to ensure complete removal of magnetic beads, falcon was placed in the magnet for another 10 min, at RT. Pure CD14⁺ monocytes were then transferred to a new falcon (leaving ~5 ml behind) and the 30 ml suspension left at this stage was topped up with 1 mM EDTA-PBS Ca²⁺/Mg²⁺ to a final volume of 40 ml. Centrifugation for 5 min, at 335 g, at RT followed and cell pellet was washed once with 1X PBS and stored at -80 °C. Prior to biochemical analysis, pellets were lysed with 50 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 270 mM Sucrose, 1% Triton + 1X protease EDTA-free/phosphatase cocktail inhibitors (Roche) added fresh), for 30 min on ice. Lysates were then centrifuged at 45,000 g, for 1 h, at 4 °C and S/N was collected and stored at -80 °C.

3.4 Isolation of plasma

Blood was collected in K₂-EDTA Vacutainer collection tubes (BD) and transferred in 15 ml falcon tubes. Centrifugation for 15 min, at 1,500 g, at 4 °C followed and S/N containing plasma was aliquoted in 2 ml cryo-vials and stored at -80 °C.

3.5 Isolation of urinary EVs

Urine (~50-200 ml) was collected from participants and tested for urinary infections. An initial centrifugation took place for 30 min, at 4,500 g, at 4 °C for debris clearance and S/N was stored at -80 °C in 50 ml falcon tubes. After quick urine thaw in a 42 °C waterbath, EVs were isolated by ultracentrifugation (Sorval Discovery, 100 SE) at 100,000 g, for 1 h at 4 °C, with 20 ml starting volume in glass tubes in T-865 fixed angle rotor (ThermoScientific). EV pellets were lysed with 50 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 270 mM Sucrose, 1% Triton + 1X protease/phosphatase inhibitors added fresh), for 1h on ice. Lysates were then centrifuged at 13,000 rpm, for 15 min, at 4 °C and S/N was collected and stored at -80 °C until further analysis.

3.6 Isolation of neuronal EVs from plasma

Freshly thawed plasma (500 μ l) was centrifuged at 3,000 g for 15 min, at 4 °C. S/N was collected and transferred to a new eppendorf where 400 μ l of ice cold 1X Dulbecco's phosphate-buffered saline (D-PBS) were added. Five μ g of L1CAM-biotinylated antibody (Anti-Hu CD171 biotin, Invitrogen eBioscience) with 1X protease EDTA-free/phosphatase cocktail inhibitors (Roche) were added and topped up with 1X D-PBS to a final volume of 1 ml. The mix was incubated O/N under rotation at 4 °C. The next day, streptavidin (SA)-agarose resin (30 μ l) was washed with 1ml 0.1% bovine serum albumin (BSA)/D-PBS and spun down at 2,500 g for 2 min, at 4 °C. S/N was discarded and procedure was repeated twice. 40 μ l of 3% BSA/D-PBS with cocktail inhibitors were added and this pre-washed resin was incubated with L1CAM-plasma mix for 2 h, at 4 °C under rotation. The resin was centrifuged at 200 g for 10 min at RT, and the S/N was transferred to a new eppendorf. The samples were subjected to two more washes with 1ml 0.1% BSA/D-PBS and then 1ml D-PBS with cocktail inhibitors took place, as described previously. For EV elution from beads, 50 μ l of 0.1M Glycine-HCl, pH 2.8 were added, vortexed briefly and shook vigorously for 5 min. Spin down at 2,500 g for 5 min, at RT followed and S/N was transferred to a new eppendorf containing 15 μ l 1M Tris-HCl, pH 8. These steps were repeated and the two eluates were combined (optional).

3.7 Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

For the quantification of total and phosphorylated LRRK2, 96-well Flat Bottom, White Microplates (Costar) were coated with 50 µl/well anti-LRRK2 antibody (ab133474 Abcam rabbit, clone c41-2, 1:2,000) diluted in coating buffer (0.168 g NaHCO₃ in 20 ml ultrapure H₂O, pH 9.4-9.6) and left O/N covered with tape, at RT. The plate was washed 3x with 1X ELISA wash buffer and 50 µl/well of samples were added in triplicates diluted in 1X 1% BSA/TBST (1:50 PBMCs, 1:100 Monocytes). Standards of recombinant human full-length WT LRRK2 (Thermo Fisher Scientific) [0.625 - 10 ng/ml] were included in each plate for normalisation. Lysates were incubated for 2 h, at 700 rpm shaking, at 37 °C. Samples were washed 3x with 1X ELISA wash buffer. For detection, horseradish peroxidase (HRP)-conjugated anti-LRRK2 (NeuroMab, N241A clone, 1:15,000) or HRP-conjugated anti-PS935 (ab133450 Abcam, UDD2, rabbit, 1:30,000) diluted in 1X 1% BSA/TBST was incubated for 1h, at 700 rpm shaking, at RT. After 3x washes, 50µl/well of ECL (Supersignal Femto, Thermo Fisher Scientific) diluted with ultrapure H₂O (Invitrogen) (1:1) was incubated for 5 min, at 700 rpm shaking, at RT covered with foil. Luminescence signal was measured using TECAN SPARK 10M microplate reader (Integration time: 10ms; Settle time: 150msec; Output: Counts). For the intrinsic LRRK2 kinase activity assay, after the 2h binding and washing step, 2x washes followed with kinase reaction buffer (+ 2 mM DTT) and a 30 min incubation with kinase reaction buffer (+ 100 µM ATP, 5 µM biotin-LRRKtide, 1X EDTA-free prot/phos inhibitors), at 700 rpm shaking, at 30 °C took place. The kinase reaction mixture was collected and transferred to PCR tubes. A plate coated with SA overnight was washed 3x and 5 µl of reaction was added together with 45 µl Stop buffer and incubated for 1 h, at 700 rpm shaking, at 37 °C. After 3x washes, it was incubated with 50 µl/well anti-phospho LRRKtide antibody (p-ERZIN/ERM (T597) Cell Signalling Technologies, 1:1,000) in 1X 1% BSA/TBST for 1h, at 700 rpm shaking, at RT. 3x washes followed and Polyclonal goat anti-Rabbit HRP (Dako, 1:30,000) diluted in 1X 1% BSA/TBST was added for 30 min, at 700 rpm shaking, at RT. After a final washing step, signal was read as described above.

3.8 Multiplex Enzyme-Linked Immunosorbent Assay (ELISA)

All reagents were prepared in 1X 1% BSA/TBST (with the samples diluted as follows: 1:3 CSF, 1:50 PBMCs, 1:100 Monocytes). Standards of recombinant human full-length WT LRRK2 (Thermo Fisher Scientific) [0.625 - 10 ng/ml] were included in each plate for normalisation. 50 µl/well of samples were added in triplicates to an antibody printed 96-well plate supplied from Quansys Biosciences. Plates had 4-printed antibody spots for LRRK2 (MC.028.83.76.242, Biolegend, ab170993 Abcam, ab172378 Abcam, ab172382 Abcam) and a Reference point. Lysates were incubated for 2 h, at 700 rpm shaking, at RT. Samples were washed 3x with 1X Quansys Biosciences wash buffer. For detection, biotinylated anti-LRRK2 (ab172378 Abcam, C41-2, rabbit, 1:2,000) diluted in 1X 1% BSA/TBST was incubated for 1.30 h, at 700 rpm shaking, at RT. After 3x washes, SA-HRP was incubated for 20 min, at 700 rpm shaking, at RT. 6x more washes followed and 50µl/well of 1:1 ECL (Supersignal Fempto, Thermo Fisher Scientific) was incubated for 5 min, at 700 rpm shaking, at RT covered with foil. Luminescence signal was measured using Quansys Q-view Imager (Exposure time 270 sec).

3.9 Western immunoblotting

To determine the protein concentration, Bradford assay using Bovine Serum Albumin standards [0.625 - 10 mg/ml] (PanReac/AppliChem) was performed in 96-well Flat Bottom, Clear Microplates (Greiner), using TECAN SPARK 10M microplate reader. 15 µg of PBMC and CD14⁺ monocyte lysates were loaded in 10-well combs (Bio-Rad) and separated by Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Bio-Rad) for ~1.5 h, at 90-120 V. In the case of urinary exosome lysates, 25 µl were loaded in each well. Gels were then transferred onto 0.45 µm Nitrocellulose membranes (Life Sciences) and transfer took place on ice for 1.5 h, at 150 V and 400 mA. Membranes were then blocked in either 5% Milk/TBST (for Rab10, Rab29, GAPDH, LRRK2, and Flotilin-1) or in 5% BSA/TBST (for pS1292-LRRK2, pT73-Rab10 and pT71-Rab29), for 1 h at RT. The primary antibodies used were as follows: Rab10 (ab237703 Abcam, rabbit, 1:1,000), Rab29 (ab199644 Abcam, rabbit, 1:1,000), Flotilin-1 (sc-133153 Santa Cruz, mouse, 1:1,000), LRRK2 (ab133474 Abcam, rabbit, clone c41-2, 1:1,000), pT73-Rab10 (ab230261 Abcam, rabbit, 1:1,000) and pT71 Rab29 (ab241062 Abcam, rabbit, 1:1,000), and pS1292-LRRK2 (ab203181 Abcam, clone MJF-19-7-8, 1:1,000), shaking overnight at 4°C. In some cases, where the membranes were probed with anti pS1292-LRRK2 antibodies, Signal Boost Immunoreaction Reagent (Sigma) was included in the antibody diluent for both primary and secondary antibody incubation steps. This reagent had been included in the antibody labelling of urinary EVs, as previously reported, in order to enhance the chemiluminescence signal. Membranes were then washed 3x for 10 min in 1X TBST and incubated with HRP-conjugated secondary antibodies (Millipore AP132P anti-rabbit or Millipore AP124P anti-mouse, 1:5,000), for 1 h at RT. 3x more washes followed and membranes were exposed to ECL and developed using an automatic film developer. Films were scanned and quantified using ImageJ, with the final Figures compiled using Adobe Photoshop.

3.10 Transmission Electron Microscopy (TEM)

For the preparation of urinary and neuronal EVs for visualization by transmission electron microscopy (TEM), the pellets were resuspended in 50 µl of 1X PBS, and 2 µl of EV suspension was removed for imaging. The samples were processed as described in Rikkert *et. al.* [194].

3.11 Statistical analysis

For statistical comparison of the different study groups, ANOVA with Tukey's post-hoc multiple comparisons were employed, or two-tailed t-tests where indicated. Analysis was carried out using GraphPad Prism software.

RESULTS

VISIT 1

4.1 LRRK2 expression, phosphorylation and *in vitro* kinase activity in immune blood cells measured by ELISA – Athens cohort

4.1.1. Total LRRK2 expression in PBMCs and CD14⁺ monocytes

It is known that LRRK2 is highly expressed in immune cells [134]. In order to quantify its expression, during the 1st visit of this study, PBMCs and CD14⁺ monocytes were isolated from blood of participants (as described in materials & methods) and LRRK2 was captured and detected with sandwich ELISA. Regardless of the cell type analysed, total expression did not differ among the groups (Fig. 7A, 7D) and this has been confirmed in other studies for iPD and even for G2019S PD [174, 175]. Here, it is the first time that LRRK2 levels have been quantified for A53T-PD compared to other forms of the disease. CD14⁺ monocytes express remarkably higher relative levels of LRRK2 (1.5 µg/ml) compared to the mixed population of PBMCs (0.5 µg/ml) and there is a very strong, significant correlation of LRRK2 expression between the two cell populations isolated from the same individual, where PBMCs with high LRRK2 expression also matched high levels in the corresponding CD14⁺ monocytes (Fig. 7G).

4.1.2 S935 LRRK2 phosphorylation in PBMCs and CD14⁺ monocytes

By measuring pS935 levels in PBMCs normalised to total LRRK2, a significant increase was found in iPD compared to HC (Fig. 7B). This finding has been also reported before in a published study from our lab from an independent cohort [174]. In contrast to this, another group has failed to detect such changes, however; they used WB as the quantification technique and this discrepancy could be due to the increased sensitivity and improvement in quantifiability afforded by an ELISA-based approach [173]. Other studies have reported reduced pS935 levels in G2019S, but pS935 levels are unaffected in cells from A53T-PD patients, as shown in this report [175]. These differences in phosphorylation between different PD groups indicate that there are distinct mechanisms in each PD condition.

Regarding pS935-LRRK2 levels in CD14⁺ monocytes, phosphorylation at this site does not change in iPD, as shown in PBMCs, nor does the kinase activity (Fig. 7E-F). This is an interesting finding as monocytes are a subset of PBMCs and among all PBMCs they express the highest LRRK2 levels. Again, levels or activity do not change in A53T-PD. These data indicate that LRRK2 is differently regulated in specific blood cell types, but the mechanisms for this need to be explored.

4.1.3 *In vitro* kinase activity in PBMCs and CD14⁺ monocytes

Despite the increase in the S935-LRRK2 phosphorylation levels in PBMCs, the *in vitro* kinase activity was unchanged, highlighting the fact that pS935 is not a direct measure of LRRK2 kinase activity, coming in agreement with other studies (Fig. 7C). Additionally, regardless of the elevated LRRK2 expression levels in CD14⁺ monocytes, the normalised intrinsic kinase activity is significantly less in this cell population compared to PBMCs, indicating that LRRK2 is not very active in this cell type (Fig. 7F). However, it would be more appropriate to compare it with another purified cell type, i.e. neutrophils. It is possible that a significant proportion of kinase activity detected in PBMC extracts is contributed by other cells than CD14⁺ monocytes, although a significant proportion of the total LRRK2 that is detected in PBMCs likely comes from this cell type. Isolated neutrophils express very high levels of LRRK2 as well and can easily contaminate preparations of PBMCs isolated using standard density gradient approaches; and it is possible that LRRK2 purified from these cell types exhibits greater kinase activity compared to CD14⁺ monocytes [129]. The mixed population of PBMCs shows alterations in normalised pS935 levels, however; the distinct cell types should be examined separately in future studies.

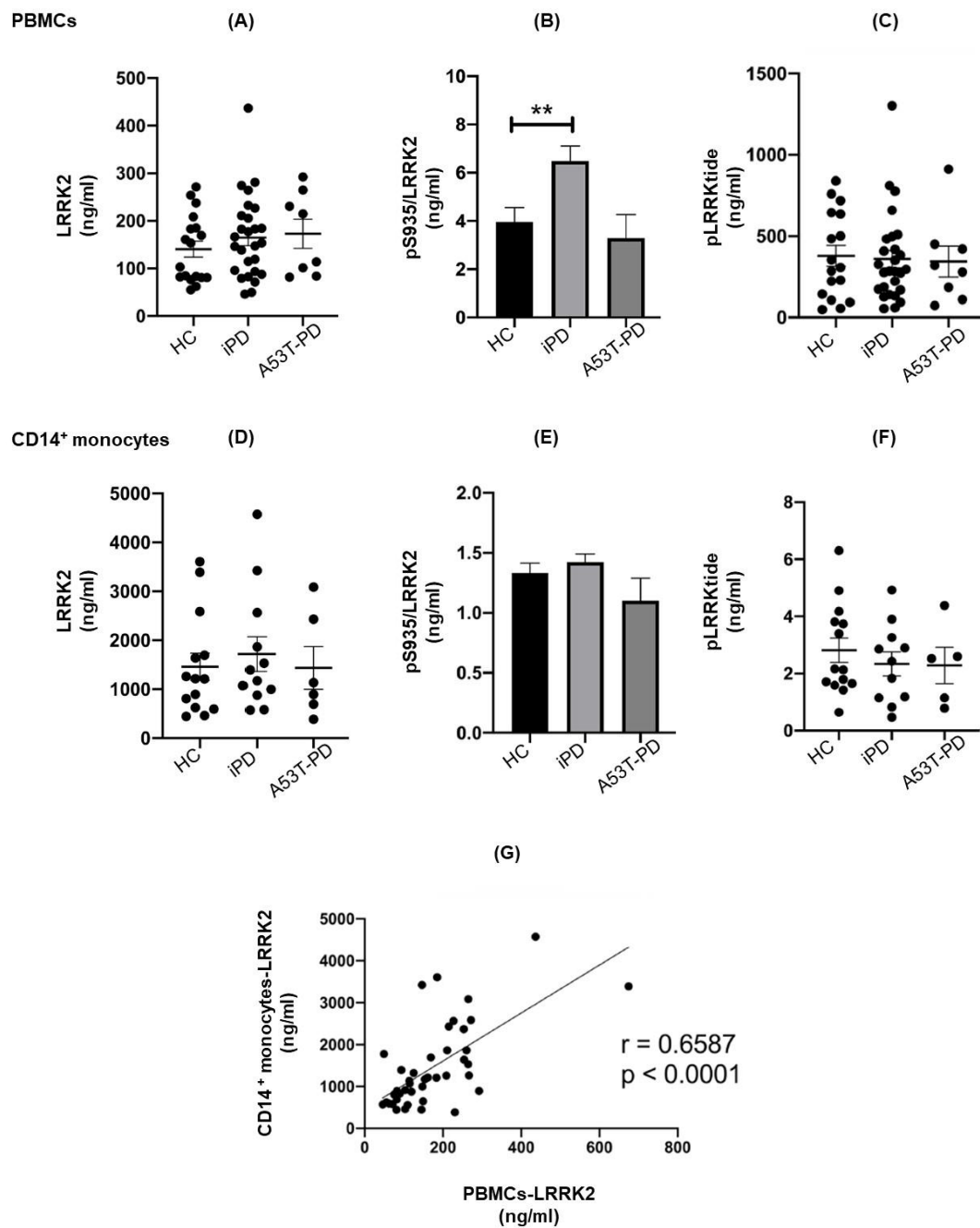


Figure 7: Quantification of total LRRK2, pS935 levels and in vitro kinase activity in immune blood cells of Athens cohort. LRRK2 levels (ng/ml) of PBMCs (A) and CD14⁺ monocytes (D) were analysed by sandwich ELISA at 1:50 and 1:100 dilutions respectively with C41-2 (1:2,000) capture and N241 (1:10,000) detection antibodies. Statistical analysis was subjected to one-way ANOVA followed by Tukey's post-hoc test (* $p < 0.05$). pS935 levels (ng/ml) normalised to total LRRK2 in PBMCs (B) and in CD14⁺ monocytes (E) were analysed by sandwich ELISA at 1:50 and 1:100 dilutions respectively with C41-2 (1:2,000) capture and pS935 (1:20,000) detection antibody. Measurements were normalised to total LRRK2 signal. In vitro kinase activity measured by quantifying the phosphorylation of LRRKtide artificial substrate peptide in PBMCs (C) and in CD14⁺ monocytes (F). Correlation of LRRK2 expression

in PBMCs vs CD14⁺ monocytes from the same participant subjects (G). Statistical analysis was subjected to one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). Error bars in all data plots represent SEM.*

4.2 LRRK2 autophosphorylation levels in immune blood cells measured by WB

4.2.1 S1292 LRRK2 autophosphorylation in PBMCs and CD14⁺ monocytes

Prior to assessment of Rab phosphorylation in clinical samples as an index of LRRK2 kinase activity, autophosphorylation of LRRK2 itself at S1292 was used to reflect a direct measure of LRRK2 function. LRRK2 migrates as two bands in the two cell types where the lower molecular band (~170 kDa) is a truncated form of the protein that lacks the N-terminal as it is not recognised by an antibody raised against this domain of LRRK2, hence; only the upper band (~286 kDa) that represents the full-length protein has been taken into consideration for result interpretation (Fig.8). While total LRRK2 expression is readily detectable, S1292 phosphorylation is undetectable, even when increasing amounts of protein extracts were loaded (Fig. 8). Increased phosphorylation at this residue has been reported in LRRK2 present in urinary EVs from both iPD as well as G2019S LRRK2 PD patients, but this was only in the presence of a Signalbooster reagent [185]. This enhancer was also used here for testing, however; as it may alter the linearity of the detection of pS1292 LRRK2 by WB in unknown ways, it was elected not to use this pharmacodynamics readout in assessing LRRK2 function in these cell types.

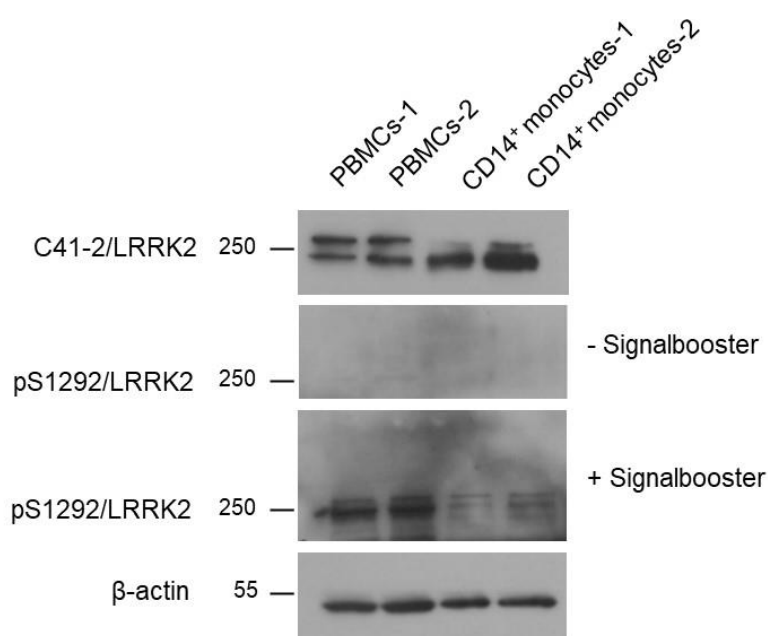


Figure 8: S1292 autophosphorylation levels in immune blood cells. 15µg of PBMC and CD14⁺ monocyte lysates were separated by SDS-PAGE and probed for total LRRK2 (C41-2) and pS1292 to test autophosphorylation levels in the absence (A) or presence (B) of a Signalbooster.

4.3 Total Rab10 and Rab29 levels and phosphorylation in immune blood cells measured by WB – Athens & CUMC cohorts

4.3.1 Total Rab10 and pT73-Rab10 levels in PBMCs

In order to address if phosphorylation of endogenous LRRK2 substrates is influenced by the different PD mutations, levels and phosphorylation of different Rab GTPases were assessed in the two cohorts as a measure of kinase activity. Firstly, Rab10 levels were quantified from PBMC extracts by WB. No differences were observed in total levels (as with total LRRK2), whereas when normalising pT73-Rab10 to total Rab10 expression, a significant increase was observed in iPD compared to HC, a finding that was replicated in both Athens and CUMC cohorts (Fig. 9A-B). To show that this phosphorylation is largely mediated by LRRK2 kinase activity, a different set of PBMCs was cultured and treated with MLi-2 kinase inhibitor at different doses and timepoints that robustly reduced both pS935 and pT73-Rab10 phosphorylation compared to DMSO treated cells (Fig. 9A-D). Additionally, in the CUMC samples, non-affected G2019S carriers could be distinguished from affected carriers as the former had significantly lower levels of Rab10 phosphorylation (Fig. 9B). In Melachroinou *et al.*, PBMC lysates from G2019S+/PD- and G2019S+/PD+ patients showed elevated *in vitro* kinase activity by ELISA, supporting the increased pT73-Rab10 levels observed here, at least concerning the affected carriers [174]. As a result, this may be a useful biomarker to monitor disease status. However; comparing these findings to clinical score outcomes (MDS-UPDRS-III, MoCA, disease duration) no significant correlations were found (Tables 5-6). As with LRRK2, no changes were observed in the A53T-PD group. As an alternative approach of quantification, levels of pT73-Rab10 were also normalised to total LRRK2 levels, where same trends were observed in iPD and G2019S+/PD+, but did not reach significance (Fig. 9C).

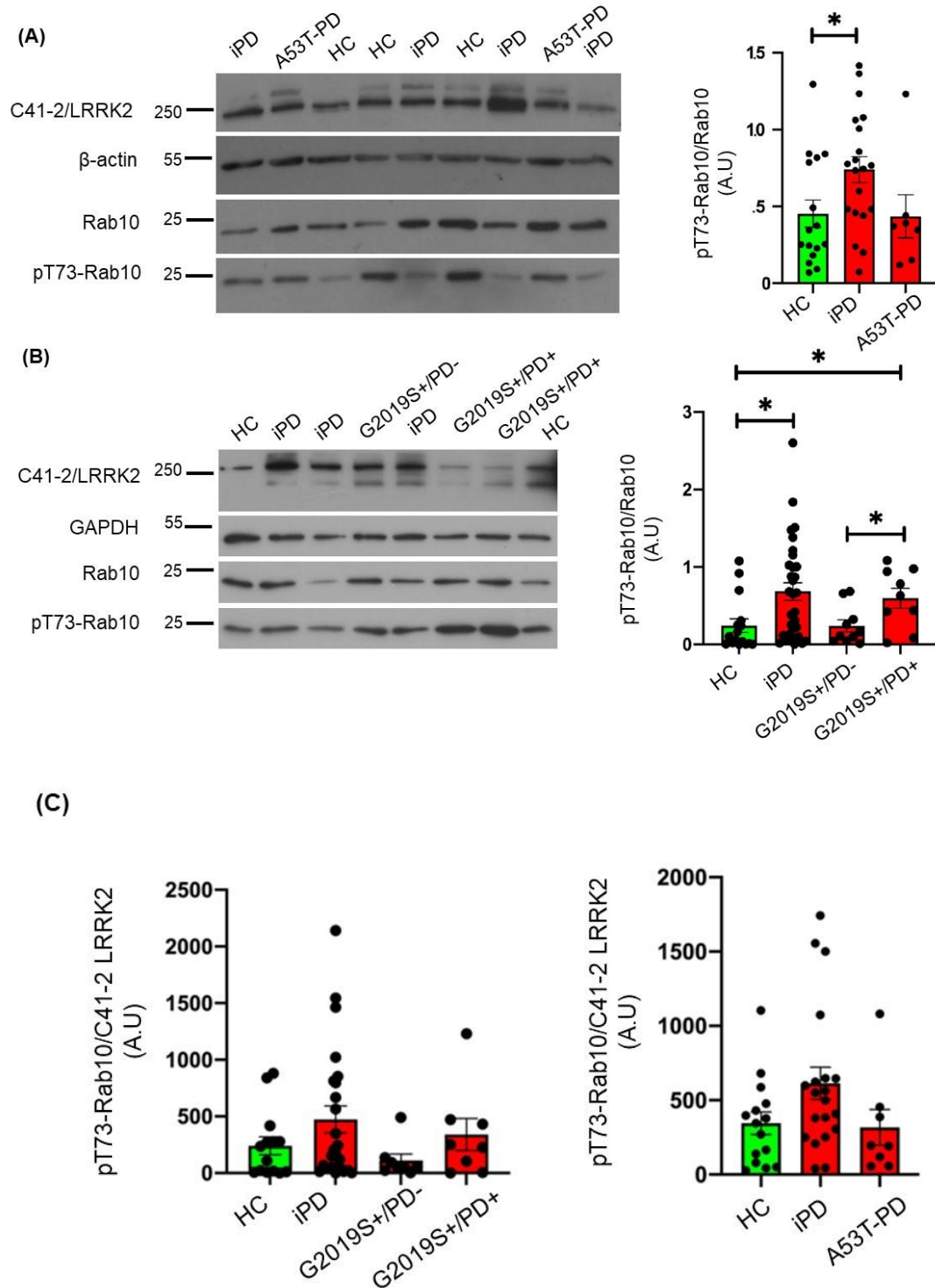


Figure 9: Rab10 phosphorylation in PBMCs from Athens and CUMC PD cohorts. Cell lysates from Athens (A) and CUMC cohorts (B) were separated by SDS-PAGE and probed for total and pT73-Rab10 levels. Band intensities (arbitrary units A.U.) were quantified and total levels were normalised to β -actin loading control and then pT73-Rab10 to the corrected total and were subjected to one-way ANOVA statistical analysis followed by Tukey's post-hoc test (* $p < 0.05$). Error bars in all data plots represent SEM. Correlation between pT73-Rab10 levels and total LRRK2 levels (C).

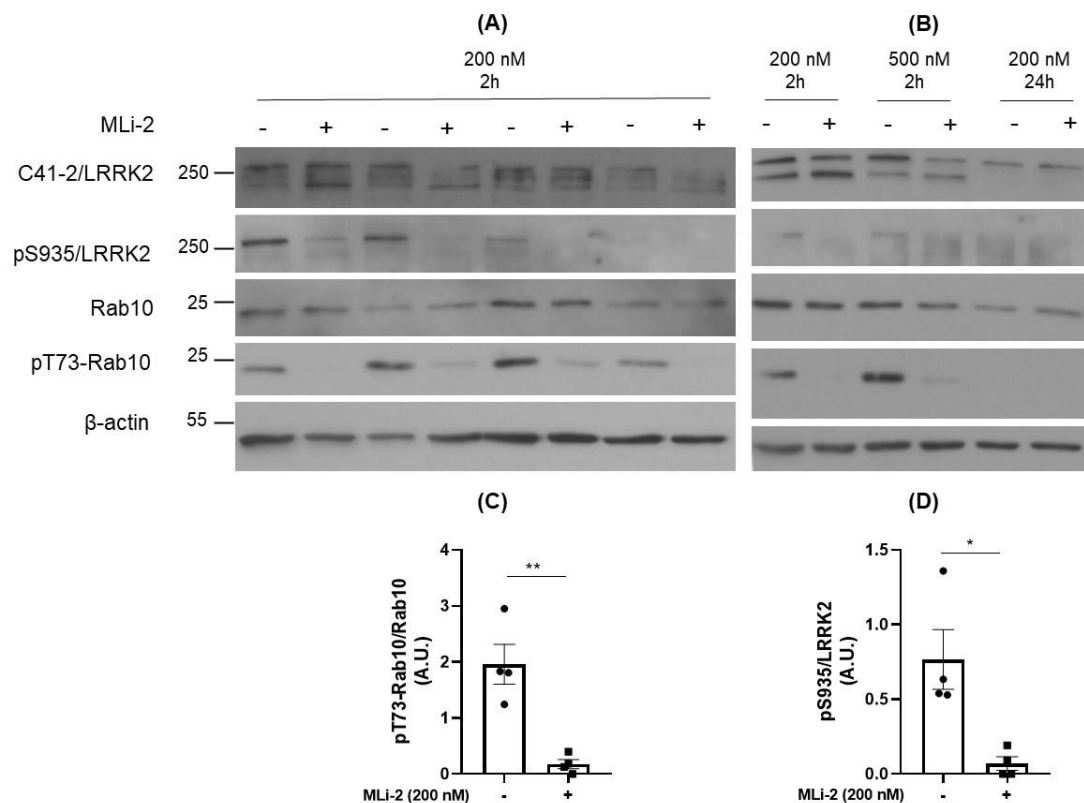


Figure 10: PBMC MLI-2 inhibitor treatments. Cells were cultured and treated with MLI-2 inhibitor at 200 nM for 2h (A) at 200 nM and 500 nM for 2 and 24h hours (B). Membranes were probed for total (C41-2) and phosphorylated LRRK2 (pS935) and total and phosphorylated Rab10 (pT73-Rab10). Lower expression in phosphorylation is observed at all doses and timepoints regarding both proteins (A-B). Quantification of pT73-Rab10 and pS935 as normalised to total Rab10 and C41-2/LRRK2 respectively after MLI-2 treatment at 200 nM for 2h (C-D).

4.3.2 Total Rab10 and pT73-Rab10 levels in CD14⁺ monocytes

CD14⁺ monocyte extracts were also analysed for Rab10 expression and levels of pT73-Rab10 phosphorylation. Contrast to what was observed in PBMCs, no differences were found among the groups in the Athens cohort (Fig. 11). Collection of CD14⁺ monocytes from the CUMC cohort for comparison was not possible, where it could possibly be expected to detect differences, at least in the G2019S patients. Again, as with ELISA results for LRRK2 and pS935, CD14⁺ monocytes are not likely the cellular source responsible for the differences observed in PBMCs.

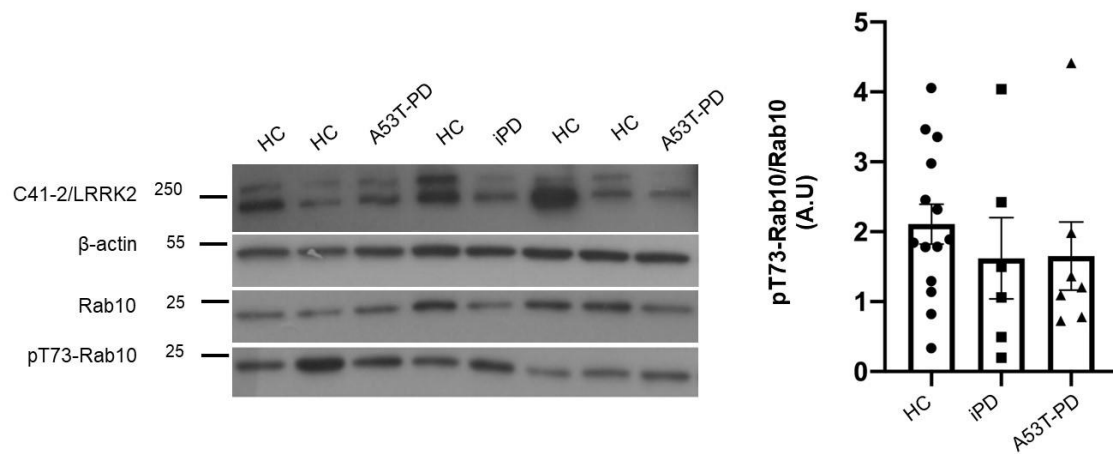


Figure 11: Rab10 phosphorylation in CD14⁺ monocytes from Athens cohort. Cell lysates from Athens cohort were separated by SDS-PAGE and probed for total and pT73-Rab10 levels. Band intensities (A.U.) were quantified and total levels were normalised to β -actin loading control and then pT73-Rab10 to the corrected total and were subjected to one-way ANOVA statistical analysis followed by Tukey's post-hoc test (* $p < 0.05$). Error bars in all data plots represent SEM.

4.3.3 Total Rab29 and pT71-Rab29 expression in PBMCs and CD14⁺ monocytes

Next, levels and phosphorylation of Rab29 were assessed. Similar to S1292-LRRK2, there are no reports in the literature examining its expression in these immune cell types, thus; the membranes were probed for total Rab29 and pT71-Rab29 to address this question. Total levels were only faintly detectable without the Signalboost reagent whereas phosphorylation was absent in both PBMCs and CD14⁺ monocytes (Fig. 12). This is surprising as Rab29 is a known phospho-substrate of LRRK2 that regulates its localisation and activity. It is evident that there are distinct mechanisms governing the interplay between LRRK2 and Rab29, as well as the reciprocal interactions between LRRK2 and other Rab's, indicating further research is needed, with more sensitive assays.

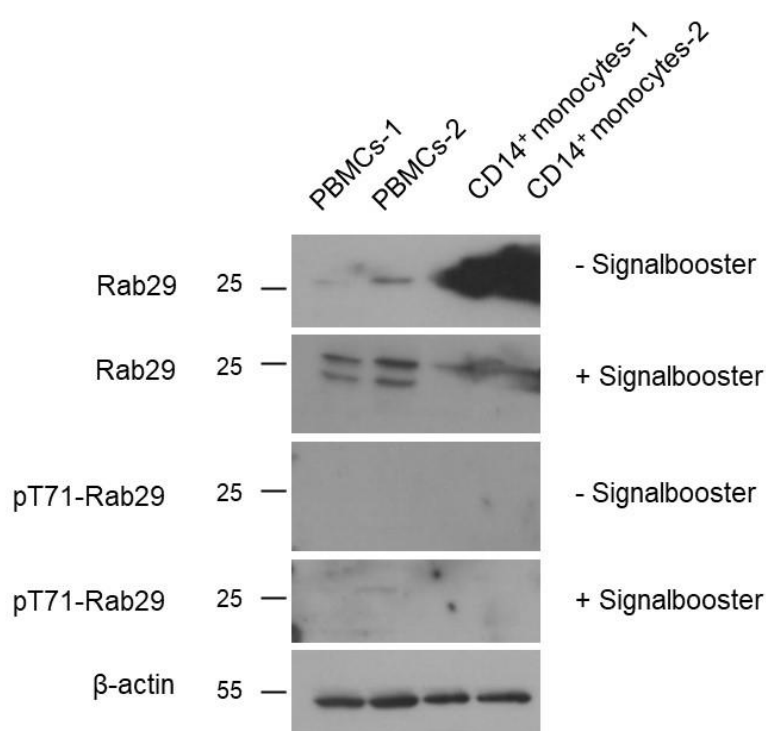


Figure 12: Rab29 expression levels in immune blood cells. 15µg of PBMC and CD14⁺ monocyte lysates were separated by SDS-PAGE and probed for total Rab29 and pT71-Rab29 to test expression levels in the absence (A) or presence (B) of a Signalbooster.

4.4 Total LRRK2 and S1292 autophosphorylation levels in urine EVs measured by WB

As discussed above, there are previous reports for total and pS1292 detection in EVs isolated from urine [183-185]. Here, however; detection of the phosphorylated form was challenging and highly variable and, in many samples, undetectable, hence; quantification was not possible (Fig. 13A). Instead, optimisation experiments in order to detect LRRK2 with ELISA were carried out, however; the chemiluminescence signal was below the sensitivity range of the assay. This may be because the 3-D structure of the protein is captured and detection is obscured because the epitope of interest may be well embedded and not accessible for binding of the antibodies used. Western immunoblots in contrast, generally assess levels of denatured linear proteins, removing any potential epitope masking. Another reason could be a “matrix effect” observed in concentrated lysates like those from EV lumens that may interfere with the signal. Nevertheless, even in the less diluted samples, detection was not possible. A representative TEM image is shown below, depicting the expected size (~125 nm) and shape of EVs recovered from urine by ultracentrifugation (Fig. 13C). Different EV markers (flotillin, TSG-101) were tested in WB analysis (13B).

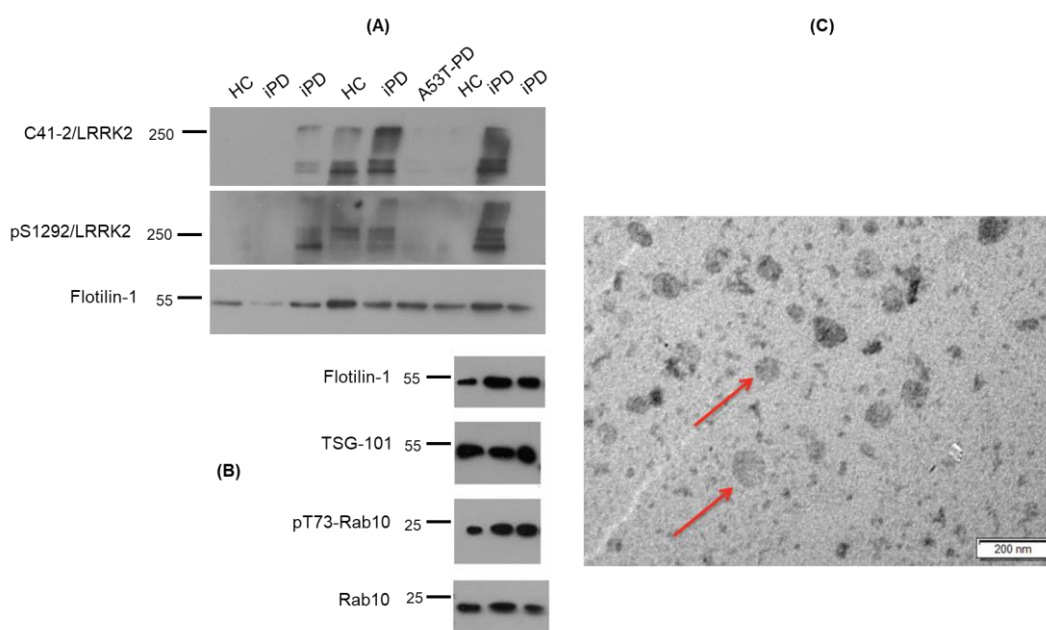


Figure 13: Expression of total and S1292 autophosphorylation in urine EVs from two cohorts and transmission electron microscopy (TEM) of urine EVs isolated by ultracentrifugation. Total and pS1292 expression of LRRK2 in urine EVs (A) and the different EV markers used in WB (B). TEM image of urine EVs at expected size (~125 nm) and shape (C).

4.5 Total Rab10 and Rab29 levels and phosphorylation in urine EVs measured by WB – Athens & CUMC cohorts

This is the first report to assess Rab GTPase phosphorylation in urine EVs. Contrast to LRRK2, it was found that both total and phosphorylated Rab10 and Rab29 are consistently detected. After normalisation, a different pattern compared to PBMCs was noted, where levels of pT73-Rab10 were unchanged in iPD in any of the two cohorts (Fig. 14A). Again, the A53T PD group showed similar levels with HC. However; this was not the case for pT71-Rab29 that is detected at significantly lower levels in both iPD and A53T-PD in the Athens cohort (Fig. 14B). This drop, however; did not correlate with any of the clinical scores reported (Table 4). Additionally, there was a trend for a slight decrease in urine EVs of iPD patients from the CUMC cohort, which only attained statistical significance following a two-tailed test and not following the ANOVA performed on the full data set (Fig. 14C). Rab10 levels were not examined in this cohort as the biofluid volume was not enough for a second set of analysis. These data could not be correlated with the increased levels of pT73-Rab10 in PBMCs (Fig. 9A-B). This may be because of the unknown cellular origin of the EVs in each sample, a profile that may even shift according to the disease or genotype status. There are multiple cell types in the PBMC population that express detectable levels of LRRK2 and Rab10, and the relevant cell type corresponding to any changes is unknown. Similarly, it is not possible in the preparations employed here, to determine the cellular source of the EVs recovered from the patient urine samples. A more targeted study of specific blood cell types and immune-isolated EVs from urine would be better suited to reveal such a correlation.

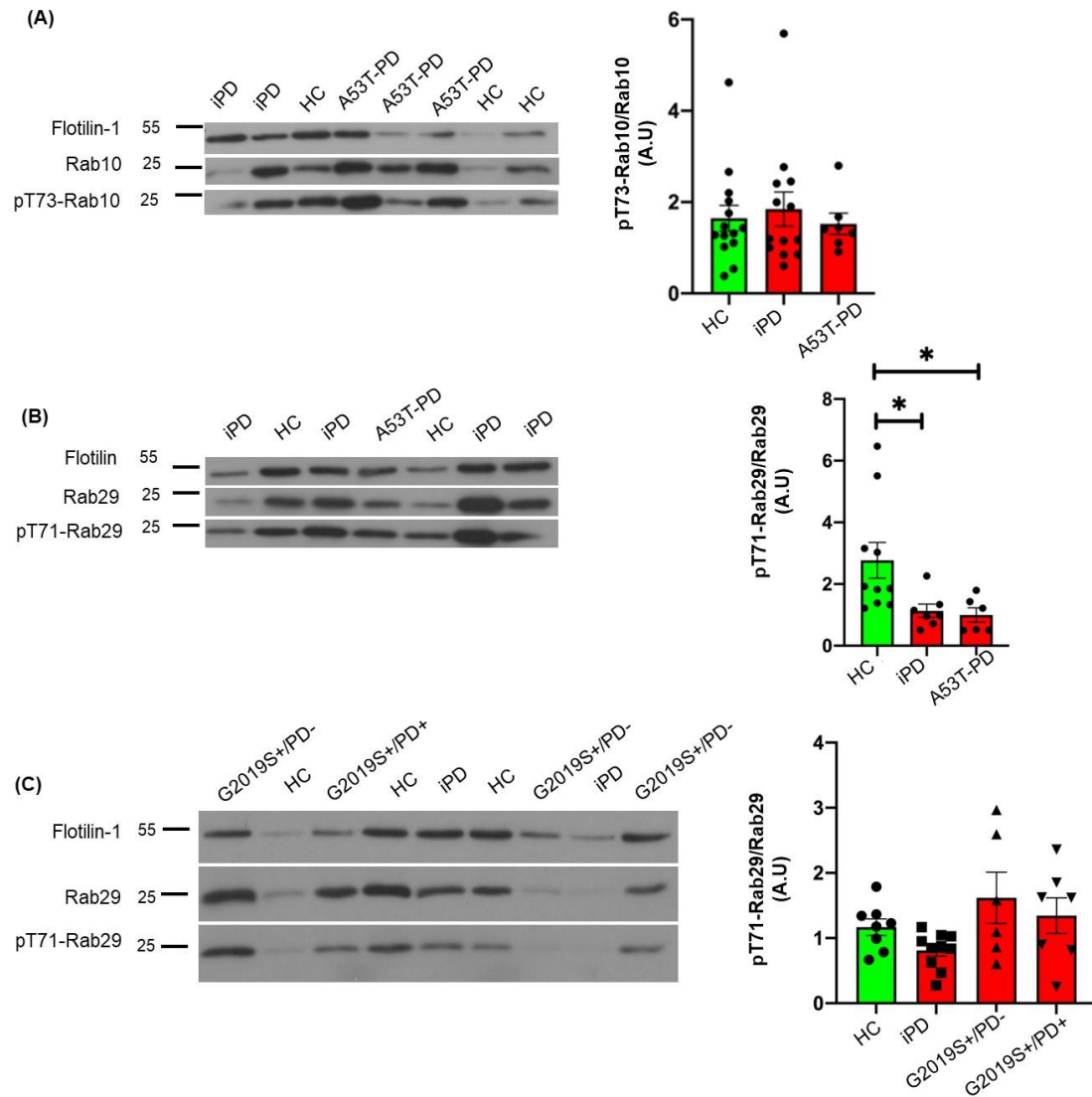


Figure 14: Rab10 and Rab29 phosphorylation in urine EVs from Athens and CUMC PD cohorts. 25 μ l of urine EV lysates from Athens (A-B) and CUMC (C) cohorts were separated by SDS-PAGE and probed for total and phosphorylated levels of Rab10 and Rab29. Band intensities (A.U.) were quantified and total levels were normalised to flotillin exosomal loading control marker and then pT73-Rab10 and pT71-Rab29 to the corresponding corrected total and were subjected to one-way ANOVA statistical analysis followed by Tukey's post-hoc test (* $p < 0.05$). Error bars in all data plots represent SEM.

All LRRK2-based biomarker outcomes are summarised in Table 3.

	Total LRRK2 (ELISA)	pS935-LRRK2 (ELISA)	<i>In vitro</i> kinase activity (ELISA)	p1292-LRRK2 (WB)	pT73-Rab10 (WB)	pT71-Rab29 (WB)
HC	Baseline	Baseline	Baseline	Detectable; not quantitative	Baseline	Baseline
iPD	Like HC	↑ ** Increase (PBMCs- 2 cohorts)	Like HC	Detectable; not quantitative	↑ * Increase (PBMCs- 2 cohorts)	↓ * Decrease (Urine EVs- 2 cohorts)
A53T-PD	Like HC	Like HC	Like HC	Detectable; not quantitative	Like HC	↓ * Decrease (Urine Evs)
G2019+/PD-	Like HC	↓ Slight decrease (PBMCs) <i>Melachroinou et. al. 2020</i>	↑ ** Increase (PBMCs) <i>Melachroinou et. al. 2020</i>	Detectable; not quantitative	Like HC	Like HC
G2019S+/PD+	Like HC	↓ Slight decrease (PBMCs) <i>Melachroinou et. al. 2020</i>	↑ * Increase (PBMCs) <i>Melachroinou et. al. 2020</i>	Detectable; not quantitative	↑ * Increase (PBMCs)	Like HC

Table 3: Summary of LRRK2-based biomarkers of both Athens and CUMC cohorts.

4.6 Neuronal EVs isolated from plasma

The isolation of neuronally-derived EVs from plasma is based on the principal of immunopurification using specific markers present on the vesicle membrane of released EVs. For example, EVs released from astrocytes are purified using GLAST-specific antibodies; and from neurons using L1CAM [195, 196]. The goal here was to isolate LRRK2 present in neuronally-derived EVs, and perform the same panel of assays as for the blood cells. Several rounds of optimization of the original protocol for the isolation of L1CAM+ EVs from human plasma have been undertaken by using various amounts of L1CAM-biotinylated antibody (1-10 μ g). A TEM image of L1CAM+ EVs isolated by direct IP is shown in Figure 15A. While the quantity in this pilot isolation is considerably less, the size distribution of visible EVs from urine or plasma appears similar (Fig. 13C, 15A). Several ELISAs were carried out to determine if there is reliable LRRK2 detection in the neuronal L1CAM-purified EVs. While variable, detection was readily possible in a few samples however; due to low recovery EV rate, analysis of the rest of the samples was not viable (Fig. 15B). As these EVs are derived from neuronal cells, a robust phosphorylation at S1292 is expected, which has been reported in EVs purified from CSF, however this modification was undetectable by WB (not shown) [184].

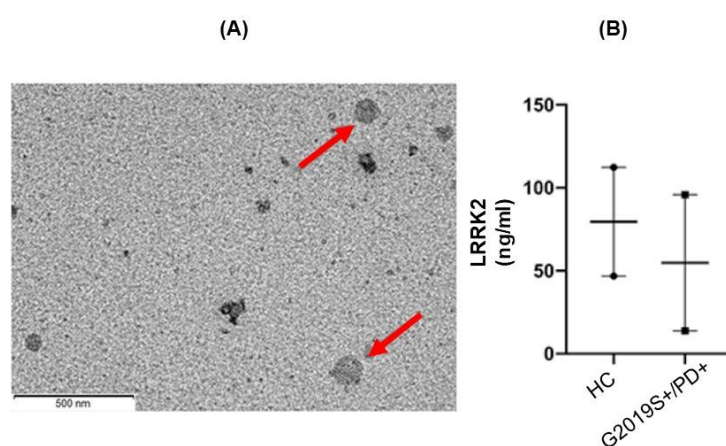


Figure 15: L1CAM+ neuronal EVs isolated from plasma by IP. (A) TEM imaging of L1CAM+ EVs of expected size and shape. (B) LRRK2 expression in extracts of 4 pilot samples of L1CAM+ EVs as quantified by ELISA at 1:10 dilution with C41-2 (1:2,000) capture and N241 (1:20,000) detection antibody.

VISIT 2

4.7 LRRK2 expression, phosphorylation and *in vitro* kinase activity in immune blood cells measured by ELISA – Athens cohort

PBMCs and CD14⁺ monocytes were again isolated during visit 2 of Athens cohort recruitment. It should be noted here that due to COVID-19 pandemic circumstances, there was a great number of participant drop-offs during this visit and the smaller sample size likely affected the analysis. Total LRRK2 levels were measured firstly in PBMCs with ELISA where no differences were found between the groups (Fig. 16A). Likewise, for pS935 LRRK2, measurements did not reach the level of statistical significance observed in the 1st visit, where levels were increased in PBMCs from iPD compared to HC (Fig. 7B). Assessment of the intrinsic kinase activity of purified LRRK2 again did not show any differences compared to HC (Fig. 7C), however; in this case, alterations were not observed in the *in vitro* kinase activity, except in the case of mutant LRRK2 carriers [174]. It is not necessarily expected to detect changes in the intrinsic kinase activity of the isolated enzyme, in the absence of activity-altering mutations, unless specific PTMs modified the conformation of LRRK2.

CD14⁺ monocytes were also collected in parallel to the PBMCs. As for the 1st visit samples, an overall marked increase was detected in LRRK2 expression, regardless of subject group, in monocytes compared to the mixed PBMC population (Fig. 16D). On an individual basis, as before, the levels of expression between monocytes and PBMCs are strongly positively correlated (Fig. 16G). With even fewer samples to compare between subject groups than were available for the PBMCs (the isolation of CD14⁺ monocytes was not feasible for every subject), it was only possible to confirm the elevated monocyte expression compared to PBMCs as well as the lack of changes in expression between patient groups. Likewise, pS935 LRRK2 levels are unchanged in monocytes from each patient group compared to HC (Fig. 16E), as is the *in vitro* phosphorylation of LRRKtide by LRRK2 purified from monocytes, however; a non-significant trend for higher intrinsic activity in LRRK2 purified from iPD monocytes was observed (Fig. 16F). In the samples collected during the initial visit, we did not observe such a trend in the *in vitro* phosphorylation of LRRKtide, however; this could reflect a general worsening of the clinical picture in iPD patients.

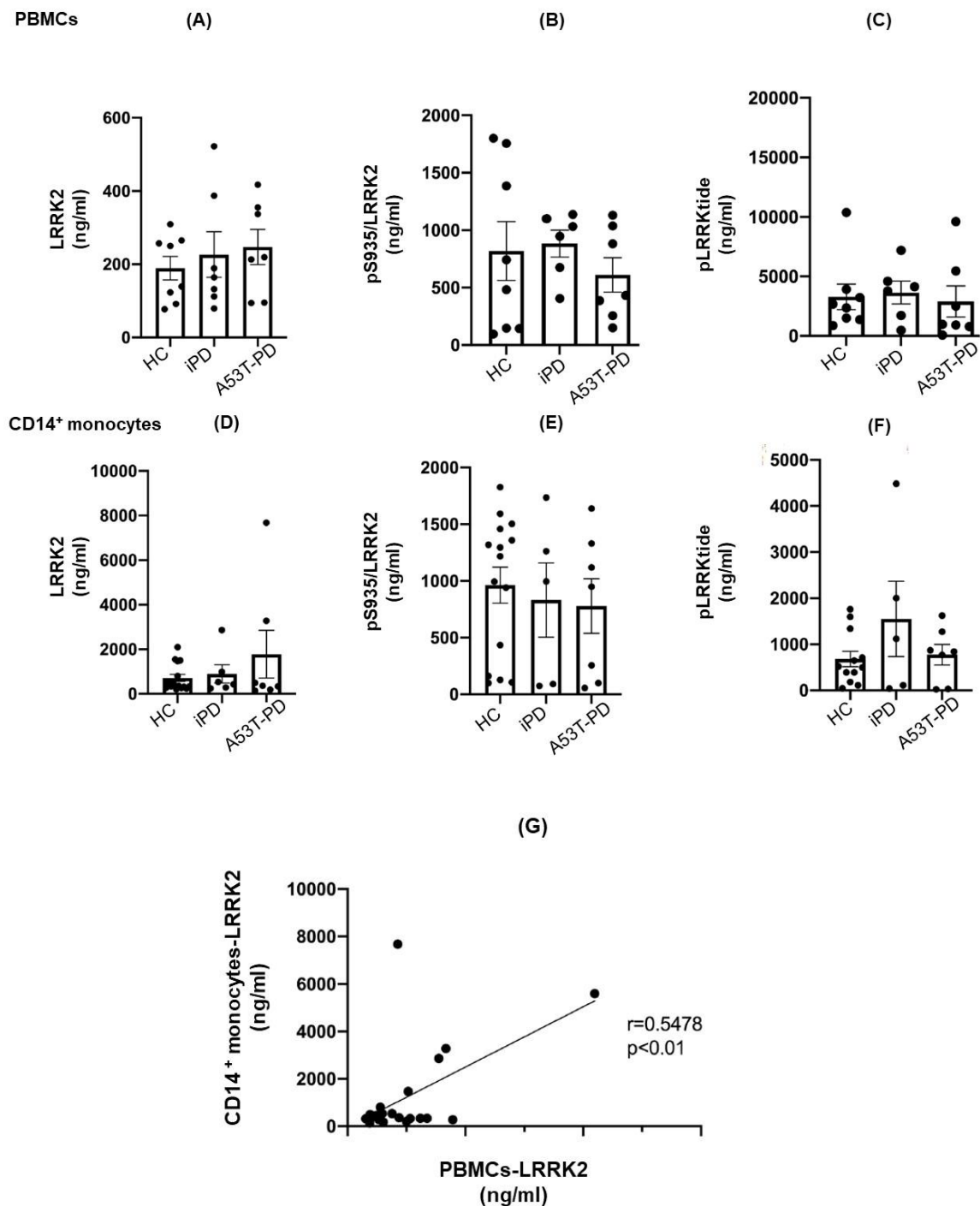


Figure 16: Quantification of total and phosphorylated LRRK2 expression in immune blood cells of Athens cohort-visit 2. LRRK2 levels (ng/ml) of PBMCs (A) and CD14⁺ monocytes (D) were analysed by sandwich ELISA at 1:50 and 1:100 dilutions respectively with C41-2 (1:2,000) capture and N241 (1:10,000) detection antibodies. pS935 levels (ng/ml) normalised to total LRRK2 in PBMCs (B) and in CD14⁺ monocytes (E) were analysed by sandwich ELISA at 1:50 and 1:100 dilutions respectively with C41-2 (1:2,000) capture and pS935 (1:20,000) detection antibody. Measurements were normalised to total LRRK2 signal. In vitro kinase activity measured by quantifying the phosphorylation of LRRKtide artificial substrate peptide in PBMCs (C) and in CD14⁺ monocytes (F). Correlation of LRRK2 expression

in PBMCs vs CD14⁺ monocytes from the same participant subjects (G). Statistical analysis was subjected to one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). Error bars in all data plots represent SEM.*

4.8 Total Rab10 and Rab29 levels and phosphorylation in immune blood cells measured by WB – Athens cohort

In the PBMCs samples collected during the 2nd planned visit, pT73-Rab10 levels failed to show the significant increase observed in visit 1 by WB in the iPD and G2019S groups of both cohorts analysed, possibly due to the reduced sample size compared to the full data set from the 1st visit (Fig 17A). Again, no difference was seen in CD14⁺ monocytes (Fig. 17B). Phosphorylated and total Rab29 were again tested by WB in both cell types but detection was unsuccessful (as was for pS1292-LRRK2).

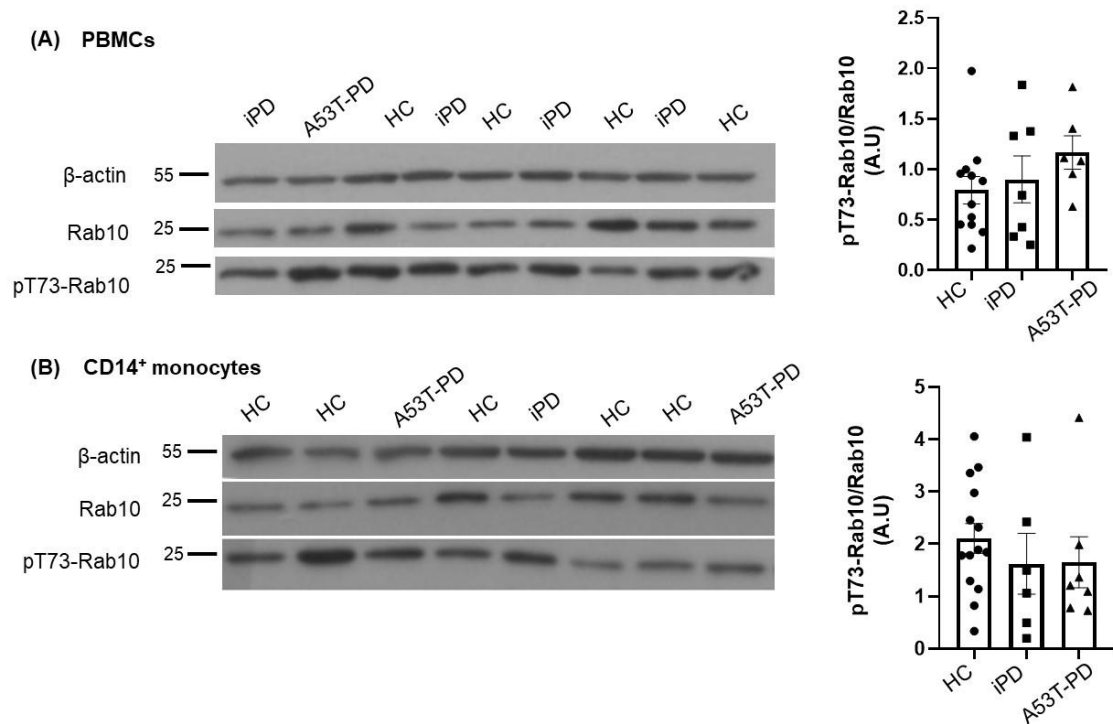


Figure 17: Rab10 phosphorylation in immune cells of Athens cohort-visit 2. PBMC (A) and CD14⁺ monocyte (B) cell lysates were separated by SDS-PAGE and probed for total and pT73-Rab10 levels. Band intensities (A.U.) were quantified and total levels were normalised to β-actin loading control and then pT73-Rab10 to the corrected total and were subjected to one-way ANOVA statistical analysis followed by Tukey's post-hoc test (* $p < 0.05$). Error bars in all data plots represent SEM.

4.9 Total LRRK2 expression and S935 phosphorylation in CSF and immune cells measured by Multiplex-ELISA

The final part of this project was to measure LRRK2 and pS935 simultaneously in the same well by using an ultra-sensitive multiplexed LRRK2-pathway focused ELISA-based array with pre-printed “spots” of the two antibodies. CSF samples were used to set up this assay that were tested at a range of different dilutions in order to find the appropriate concentration. Total LRRK2 and pS935 levels were measured in the same well with the most concentrated samples (sample dilution, 1:3) giving the more robust readings and this robustness dropped linearly with the higher dilutions (1:9, 1:27) (Fig. 18A-B). Also, while treating the samples with MLI-2 LRRK2 kinase inhibitor, pS935 levels dropped significantly, as expected (Fig. 18C). Surprisingly, detection of LRRK2 was not possible in the PBMCs or CD14⁺ monocytes tested. This was unexpected, as there is consistent and robust signal in the single-plex array, thus; further optimisation steps are necessary to resolve this issue for future studies.

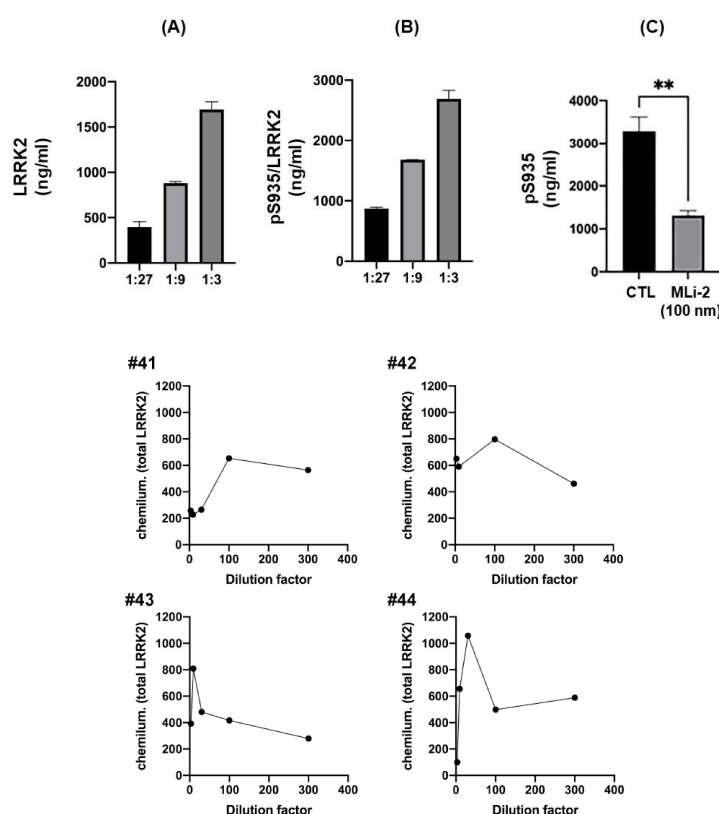


Figure 18: Quantification of total LRRK2 and pS935 levels in CSF. Total LRRK2 (A) and pS935 (B) expression measured with the different dilutions (1:3, 1:9 1:27). A robust reduction in the pS935 levels is seen with MLI-2 kinase inhibitor at 100nM (C). Total LRRK2 levels measured in four CSF samples (#41-44) at different dilutions (1:100-1:400).

4.11 Demographic/Clinical scores and correlation with LRRK2 biomarkers.

The clinical scores obtained for the full visit 1, as well as the limited visits completed during the 2nd year of the study are presented in Tables 4-6. With the major caveat of fewer subjects in the 2nd visit, compared to the 1st, due to the difficulties encountered as a result of the global health crisis; in the iPD group, a general (non-significant) worsening of their motor function is observed, as reflected in the poorer score on the MDS-UPDRS (Tables 4-5); and an accompanying increase in their symptomatic management with L-DOPA – reflected in an increase in LEDD. The A53T PD group in contrast, required a smaller increase in LEDD, compared to iPD patients, however; displayed a slight improvement in their motor function score on the MDS-UPDRS-III scale (Table 5). This could be due to a greater response to the dose management efforts during the time between the 1st and 2nd visits. Alternatively, this could be explained in part by the better response to L-DOPA generally observed in younger PD patients, which is the case for the early onset A53T PD group. A significant correlation has been previously observed between elevated pS935 LRRK2 levels and poorer performance on the MoCA cognitive test, particularly within iPD patients [174]. A similar trend was observed in samples obtained during the 1st clinical visit, however; this was not observed in the few follow-up samples collected in the 2nd planned visit (Table 5). It is likely that this would be confirmed in a larger sample.

	HC	iPD	A53T-PD	p value	Statistical tests
Sex (M:F)	7:9	14:6	4:4	ns	
Age	55 (36-75)	72 (47-85)	51 (30-73)	ns	
Age at onset	na	66 (42-80)	46 (27-68)	p=0.0011	MWU (iPD vs A53T-PD)
LEDD	na	629 (0-1865)	758 (150-1500)	ns	MWU (iPD vs A53T-PD)
UPDRS-III	0	30 (0-91)	31 (9-53)	ns	MWU (iPD vs A53T-PD)
MoCA	26 (17-30)	19 (4-26)	26 (16-30)	p=0.0025 (HC vs iPD) ns (HC vs A53T PD)	MWU
H&Y	0	2 (0-4)	2 (1-3)	ns	MWU (iPD vs A53T-PD)

Table 4: Summary of demographics and clinical scores of Athens cohort during visit 1.

	HC	iPD	A53T-PD	p value
Sex (M:F)	7:8	8:0	4:4	p=0.053
Age	51 (43-60)	67.5 (58-78)	53.5 (45-58)	p=0.003
Age at onset	na	63 (54-73)	45 (40-52)	ns
LEDD	na	659 (477-1173)	653 (236-994)	ns
UPDRS-III	0	21.5 (14-28)	16.5 (14-63)	p<0.001
MoCA	29 (28-30)	24 (19-26)	26 (17-29)	p=0.012
H&Y	0	2 (1-2)	2 (1-3)	p<0.001

Table 5: Summary of demographics and clinical scores of Athens cohort during visit 2.

	HC	iPD	G2019S+/PD-	G2019S+/PD+	p value	Comparisons
Sex (M:F)	8:12	18:12	4:5	4:5	ns	
Age	68.3 (57-85)	67.5 (45-86)	57.1 (38-69)	74 (64-90)	p<0.01-0.05	G2019S+/PD- vs all groups
Age at onset	na	60.5 (27-84)	na	64.6 (54-83)	ns	
LEDD	na	466.3 (0-1520)	na	631.3 (300-1625)	ns	
UPDRS-III	0	18.9 (5-46)	0	20.6 (12-28)	ns	
MoCA	27.8 (24-30)	26.4 (15-30)	28.4 (27-30)	26.5 (22-29)	p<0.05	iPD vs G2019S+/PD-

Table 6: Summary of demographics and clinical scores of CUMC cohort.

DISCUSSION

The overall goals of this work were to further develop a panel of biomarkers chosen from within known LRRK2 signalling pathways and to determine if any correlations exist with specific PD patient populations. The design included a semi-longitudinal study comprised of two visits, with biofluid collection six months apart from each visit. To our knowledge, it is the first to investigate whether LRRK2 levels or activity are changed in multiple biofluid samples in PD patients harbouring the A53T mutation in α -synuclein. As mentioned before, there is evidence in *in vitro* and *in vivo* models that LRRK2 and α -synuclein are acting in a common pathway, hence; examining such a relationship in human samples would be important for biomarker stratification. Patients of iPD and G2019S affected and non-affected carriers were also recruited, making up two cohorts (Athens & CUMC) that were analysed separately. LRRK2 kinase activity, its phosphorylation and phosphorylation of LRRK2 substrates were measured in PBMCs, CD14⁺ monocytes and urinary EVs. Isolation and LRRK2 detection in neuronal EVs from plasma of a few individuals was also possible, however; this stage needs further optimisation. Additionally, total LRRK2 and pS935 levels were measured simultaneously in a single well of a Multiplex ELISA, firstly in CSF from a small cohort of healthy individuals and then in the remaining PBMCs and monocytes from the two PD cohorts. It is critical to determine if changes in LRRK2 can ultimately be used as a reliable marker for disease progression more broadly in PD and results should be evaluated in multiple ways using various techniques.

In the present study, no differences were found in total LRRK2 expression levels (ng/ml) in PBMCs and CD14⁺ monocytes from any of the groups in the Athens cohort using a sandwich ELISA, an outcome that comes in agreement with the literature. The relative levels of LRRK2 expression in CD14⁺ monocytes were ~10X greater than those in PBMCs of the same individuals with a positive correlation in the two cell types. It has been stated elsewhere that among PBMCs, monocytes express very high levels of LRRK2, a finding replicated in this study as well [134]. The same outcome was found in the CUMC cohort, where total levels did not differ between HC and G2019S affected and non-affected carriers as well as iPD patients [174]. Interestingly, a significant increase in pS935 levels was detected in PBMCs of both cohorts in iPD compared to HC [174]. Another group failed to detect such change, however; they used WB for their analysis, which is a semi-quantitative method and may explain this discrepancy [173].

There is a decrease observed in the levels of pS935 in G2019S carriers manifesting PD compared to non-manifesting carriers or iPD whereas total levels

remained unchanged, implying that it is a promising marker that may be used to discriminate disease status [174, 175]. In cellular and animal models, on the G2019S-LRRK2 background, S935 phosphorylation is reduced to some extent but not as strongly as with R1441C, hence; these findings indicate that there may be distinct mechanisms regulating this PTM in the different PD genotypes (iPD compared to G2019S PD but also between affected and non-affected carriers). A disadvantage of studying PBMC population is the different cellular subtypes present including lymphocytes (B-cells, T-cells, NK cells), monocytes (CD14, CD16) and dendritic cells that could mask any alterations, thus; more cell-specific studies are necessary. Expression levels of LRRK2 are very high in monocytes and it has been shown before that CD16⁺ monocytes, B-cells and T-cells of PD patients express higher levels of LRRK2 compared to healthy individuals [134]. Thus; the aggregate pS935 increase observed here in PBMCs could come from any of these cells. To clarify this, CD14⁺ monocytes were analysed as the sub-population, however; no differences were noted. This could mean that another cell type is responsible for this increase or that the smaller monocyte sample size could not reach significance. Further studies are necessary to clarify this and more subpopulations need to be tested.

Kinase activity of LRRK2 in PBMCs and CD14⁺ monocytes of the Athens cohort was measured in an *in vitro* phosphorylation assay using LRRKtide as the artificial substrate. Since monocytes express much higher LRRK2 levels, it was presumed that they would also show higher activity. Surprisingly, the relative levels (ng/ml) were significantly lower compared to PBMCs, implying that LRRK2 is not very active in this cell type, despite its abundance, for unclear reasons at the moment. Additionally, no difference in activity was shown between the groups, even though pS935 levels were higher in iPD patients, confirming that this phosphorylation although indirectly regulated by kinase activity cannot be used as a direct measurement. In the CUMC cohort, as expected, G2019S affected and non-affected carriers showed higher kinase activity, an increase that was only evident in males [174].

Another measure for direct kinase activity is assessing S1292 autophosphorylation. Unfortunately, there are no commercially available antibodies suitable for ELISA; instead, WB was used for these analyses. It was revealed that this residue is of low stoichiometry in PBMCs and CD14⁺ monocytes, a finding that is in accordance with animal tissue studies where detection is also below sensitivity range in WT animals and only seen in BAC transgenic mice overexpressing LRRK2 [80, 197]. Detection was only possible after probing with a SignalBoost reagent where no observable differences were exhibited between subject groups, however; this enhancer may alter the linearity of the result, and was not taken into account for

analysis. Total LRRK2 and pS935 levels were also quantified and no differences were found in any of the groups. As a result, pS935 levels are no different when analysed with WB according to this and another study, nevertheless can be discriminated with the much more sensitive ELISA [173].

Two major Rab GTPases were examined in this study. A significant elevation of phosphorylated Rab10 was confirmed in PBMCs of iPD patients compared to A53T PD patients and HC in the Athens cohort and this was also replicated in the analysis of the CUMC cohort, strengthening this finding. It is well established in *in vitro* experiments that G2019S overexpressed cells have increased levels of pT73-Rab10. This was also confirmed here in the human PBMCs where G2019S affected carriers showed an increase similar to the iPD group. There was also discrimination between affected and non-affected carriers, with the latter having levels close to HC. From these data, the increased pRab10 is linked more to disease status. No correlation was found between Rab10 and LRRK2 levels, indicating that their expression regulation is independent. It is known from cellular models that phosphorylation of LRRK2 substrates is an indirect measure of LRRK2 kinase activity. However; the increase in Rab10 phosphorylation observed in the iPD group was not reflective of an increase in the enzyme's activity here, even though pRab10 levels were abolished with MLI-2 kinase inhibition. This could be because although Rab10 phosphorylation largely depends on LRRK2, it is not an exclusive substrate and potentially other kinases, as well as the activity of phosphatases are implicated in this process. Further investigation of other markers in the LRRK2 pathway is warranted, i.e., examine the expression of PPM1H phosphatase of Rab10 and assess whether its levels are decreased in the disease, resulting in the higher Rab10 phosphorylation observed here. CD14⁺ monocytes had similar phosphorylation levels among the groups, again implying that any changes observed come not from these cells or more samples are needed to reach significance. Total Rab29 levels were hardly detectable in any of these blood cells whereas phosphorylation was completely absent, even when very high amounts of protein were loaded or the Signalbooster was used. The reason for this is unclear at this point, however; similarly, with S1292, it is evident that the profile of LRRK2 substrates, as well as their regulation, exhibits a cellular sub-type dependency and for those it appears to not be prominent in peripheral immune cells.

To show that phosphorylation of these substrates is mainly LRRK2-dependent, PBMCs were treated with MLI-2 kinase inhibitor. Levels of Rab10 phosphorylation dramatically dropped or were completely diminished after 2-24 hours of 200-500 nM *ex vivo* inhibition treatment. Although it is known that Rab GTPases are not exclusive substrates of LRRK2 and cannot be used as a direct measure of the protein's kinase

activity, hopefully this study defines the critical next steps for inclusion/exclusion in potential clinical trials of LRRK2 inhibitors, as the pharmacodynamic measures of target engagement seem promising in monitoring patients under medication.

This is the first report (published at the same time as Wang *et. al.* 2022) to measure Rab phosphorylation in urine EVs [198]. It was known that these vesicles contain high levels of LRRK2 as well as pS1292, however; other groups have been unable to detect Rab10 or Rab29. As shown here by various exosomal markers (flotillin-1 and TSG-101), isolation was successful and vesicles were of the expected size (~125 nm) validated by TEM. Contrast to PBMCs, no changes in phosphorylation of Rab10 were found in any of the groups of Athens cohorts when normalised to total Rab10 and flotillin-1, although there was a trend for higher levels in the iPD group. Interestingly, Rab29 is very highly expressed in these vesicles (in contrast to PBMCs/CD14⁺ monocytes) and pT71-Rab29 is significantly lost in the iPD group of both cohorts and in the A53T group of Athens cohort when compared to HC. Recently, it was reported that overexpression of Rab29 can trigger the recruitment of LRRK2 to intracellular membranes and the TGN leading to its activation, suggesting a regulatory cycle between LRRK2 activity and Rab29 [24]. The loss of this phosphorylation in urine EVs from iPD and A53T-PD patients, releasing its inhibition, would be consistent with increased activation of LRRK2 (e.g. increased pT73-Rab10 in the iPD group). While the increased pT73-Rab10 in the iPD group in urine EVs was not significant and *in vitro* kinase activity in urine EVs was not possible to be measured, the reduction in Rab29 phosphorylation is the first indication of possible changes in LRRK2 activation in clinical samples from PD patients carrying the A53T α -synuclein mutation and bolsters the notion that LRRK2 function plays an important role in PD in general. LRRK2 and pS1292 were detectable in a few samples but had great variation with background at the higher molecular weights, thus; were not included in the normalisation analysis. As stated before, according to West *et. al.*, normalisation of pS1292 to total LRRK2 was possible and showed significant elevation in iPD and G2019S-LRRK2 patients using WB as a technique, like here [183, 185]. This discrepancy between the two studies is possibly due to the Signalbooster used in their experiments. A limitation with urine EVs is the high variability of the recovered pellet among human samples that depends on the composition and amount of urine collected from each individual that may affect the analysis and also that their source of origin is hard to determine, and it is unknown if that changes during the disease course. It was not possible to measure Rab GTPases in sandwich ELISA and replicate/compare with WB results in any of the samples as there are no commercially available antibodies suitable for this technique. Furthermore, it was possible to demonstrate by TEM

L1CAM+ EVs from plasma as a neuronal marker indicative of successful isolation by IP. LRRK2 was detected in these EV extracts in a few samples, however; the reproducibility of this isolation method needs to be improved. It is important to develop strategies to monitor the expression of proteins implicated in PD by using neuronal EVs as a non-invasive tool since their cargo represents the brain's environment, which is otherwise hard to study.

Finally, it was possible to measure total and pS935 LRRK2 in a few CSF samples by Multiplex-ELISA. This is an important first step, which indicates the potential of this approach that, if successful, could significantly facilitate LRRK2 biomarker development. On the contrary, detection of pS935 or total LRRK2 was not possible in the PBMCs or CD14⁺ monocytes tested. This was unexpected, as there is consistent and robust signal in the single-plex array with these cell types. Lack of success could be attributed to the conditions used (i.e. dilution of samples and antibodies), which were identical to those in the single-plex ELISA but may in turn not be suitable for the multiplex assay. Further optimisation is necessary to resolve this issue. A challenge that was anticipated with this assay is sensitivity issues in specific sample matrices. This is predicted to be an issue in CSF, but there are a number of potential solutions, including assessing certain targets as a *direct ELISA*, or alterations in the sample preparation. A more serious potential issue concerns cross-reactivity between certain protein targets, e.g. LRRK2 and Rab10. In light of a recent description of Rab binding sites within the ARM region of LRRK2, a careful selection of LRRK2 antibodies (e.g. clone C41-2's epitope maps to the LRR domain of LRRK2 and clone's MC.028.83.76.242 maps to the C-term region) could minimize potential epitope interference by nearby-binding proteins. Further sample preparation techniques could be employed to potentially disrupt such protein interactions, without altering epitope binding. In addition, the multiplexed technology to be employed in this assay, involving the microprinting of antibody "spots" within a single well of a 96-well ELISA plate, offers further optimization steps, including varying the antibody concentration within the micro-spot as well as the "spot" size itself. It is important to note, however; that in the case of the multiplexed assay development protocol employed by Quansys, all validation procedures are performed on the full array simultaneously, rather than one target at a time.

Taking the above into account, different biofluids of the same individual show similarities and differences in the expression patterns and activity of LRRK2 and their Rab GTPase substrates, hence; different PD groups may have distinct regulatory mechanisms and each type should be examined separately. For example, Rab10

phosphorylation is higher in PBMCs of both iPD and G2019S PD but this change is not evident in their urine EVs. Also, the lack of significant increases in A53T PD patients suggests that there are multiple PD progression pathways, and rates of clinical decline, depending on the form of the disease. This is an earlier onset form of the disease that may show transient changes at an earlier timepoint. The number of participants was lower in this group, as it is very hard to find volunteers with this mutation and this may have masked a potential increase. It is known that Rab10 phosphorylation is higher in brains of iPD patients but no studies at this point have assessed A53T or G2019S mutations. Neuronally derived EVs would be a useful tool to detect such changes and compare them with post-mortem tissue analyses. Here, although sufficient progress was made in isolating these vesicles from plasma, optimisation and analysis is still ongoing. Rab29 is highly expressed and regulated similarly in urine EVs of iPD and A53T-PD but differently in G2019S patients according to presented data, although absent (low expression) in blood cells. Furthermore, the conditions within a cellular environment are different than those occurring in an experimental assay. For example, *in vitro* kinase activity could not discriminate G2019S affected from non-affected carriers, however; this was evident with phosphorylation of Rab10. This implies that pT73-Rab10 levels may predict disease status but this is not true for the intrinsic activity of the enzyme. Also, total LRRK2 levels do not change with disease status but PTMs such as pS935 and kinase activity are higher in iPD and G2019S PBMCs. Although Rab GTPases are major substrates of LRRK2, other kinases/phosphatases are also responsible for their regulation, (e.g., PPM1H) that may also differ in the different PD forms and could explain some of the differences observed here, thus; these pathways should also be taken into consideration in future studies.

Unfortunately, due to unexpected circumstances (COVID-19 pandemic), many volunteers dropped-off during the 2nd visit of this study and a lower number of participants were recruited compared to the first visit. The majority of changes in biofluids observed in visit 1 analysis could not be replicated in visit 2. It was only possible to confirm the elevated monocyte expression compared to PBMCs as well as the positive correlation of LRRK2 levels in PBMCs and CD14⁺ monocytes from the same participant subjects. Nevertheless, significant changes in values with each subsequent sample collection may not have been observed in this study, as the duration was likely too short to observe significant changes in affected patients, however; this would serve as a useful gauge of assay stability. Despite the obstacles encountered in the 2nd year, these findings have identified several interesting features

of LRRK2 in peripheral blood cells, and as a potential discriminating factor for different PD cohorts. A longitudinal study with sufficiently large patient groups assessing multiple markers is critically needed to address these questions.

REFERENCES

1. de Lau, L.M. and M.M. Breteler, *Epidemiology of Parkinson's disease*. Lancet Neurol, 2006. **5**(6): p. 525-35.
2. Gasser, T., *Molecular pathogenesis of Parkinson disease: insights from genetic studies*. Expert Rev Mol Med, 2009. **11**: p. e22.
3. Blandini, F., et al., *Functional changes of the basal ganglia circuitry in Parkinson's disease*. Prog Neurobiol, 2000. **62**(1): p. 63-88.
4. Jankovic, J., *Parkinson's disease: clinical features and diagnosis*. J Neurol Neurosurg Psychiatry, 2008. **79**(4): p. 368-76.
5. Kalia, L.V. and A.E. Lang, *Parkinson's disease*. Lancet, 2015. **386**(9996): p. 896-912.
6. Poewe, W., et al., *Parkinson disease*. Nat Rev Dis Primers, 2017. **3**: p. 17013.
7. de Rijk, M.C., et al., *Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group*. Neurology, 2000. **54**(11 Suppl 5): p. S21-3.
8. Cosgrove, J., J.E. Alty, and S. Jamieson, *Cognitive impairment in Parkinson's disease*. Postgrad Med J, 2015. **91**(1074): p. 212-20.
9. Postuma, R.B., et al., *MDS clinical diagnostic criteria for Parkinson's disease*. Mov Disord, 2015. **30**(12): p. 1591-601.
10. Ishihara, L.S., et al., *Estimated life expectancy of Parkinson's patients compared with the UK population*. J Neurol Neurosurg Psychiatry, 2007. **78**(12): p. 1304-9.
11. Delamarre, A. and W.G. Meissner, *Epidemiology, environmental risk factors and genetics of Parkinson's disease*. Presse Med, 2017. **46**(2 Pt 1): p. 175-181.
12. Wu, Q., et al., *Chronic mild stress accelerates the progression of Parkinson's disease in A53T α -synuclein transgenic mice*. Experimental Neurology, 2016. **285**: p. 61-71.
13. Howells, F.M., et al., *Stress reduces the neuroprotective effect of exercise in a rat model for Parkinson's disease*. Behavioural Brain Research, 2005. **165**(2): p. 210-220.
14. Chang, D., et al., *A meta-analysis of genome-wide association studies identifies 17 new Parkinson's disease risk loci*. Nat Genet, 2017. **49**(10): p. 1511-1516.
15. Deng, H., P. Wang, and J. Jankovic, *The genetics of Parkinson disease*. Ageing Res Rev, 2018. **42**: p. 72-85.
16. Polymeropoulos, M.H., et al., *Mutation in the alpha-synuclein gene identified in families with Parkinson's disease*. Science, 1997. **276**(5321): p. 2045-7.
17. Singleton, A.B., et al., *alpha-Synuclein locus triplication causes Parkinson's disease*. Science, 2003. **302**(5646): p. 841.
18. Zimprich, A., et al., *Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology*. Neuron, 2004. **44**(4): p. 601-7.
19. Paisán-Ruiz, C., et al., *Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease*. Neuron, 2004. **44**(4): p. 595-600.
20. Kitada, T., et al., *Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism*. Nature, 1998. **392**(6676): p. 605-8.
21. Bonifati, V., et al., *Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism*. Science, 2003. **299**(5604): p. 256-9.
22. Valente, E.M., et al., *Hereditary early-onset Parkinson's disease caused by mutations in PINK1*. Science, 2004. **304**(5674): p. 1158-60.
23. Liu, Z., et al., *LRRK2 phosphorylates membrane-bound Rabs and is activated by GTP-bound Rab7L1 to promote recruitment to the trans-Golgi network*. Hum Mol Genet, 2018. **27**(2): p. 385-395.

24. Purlyte, E., et al., *Rab29 activation of the Parkinson's disease-associated LRRK2 kinase*. *Embo j*, 2018. **37**(1): p. 1-18.
25. Kalinderi, K., S. Bostantjopoulou, and L. Fidani, *The genetic background of Parkinson's disease: current progress and future prospects*. *Acta Neurol Scand*, 2016. **134**(5): p. 314-326.
26. Nalls, M.A., et al., *Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease*. *Nat Genet*, 2014. **46**(9): p. 989-93.
27. Satake, W., et al., *Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease*. *Nat Genet*, 2009. **41**(12): p. 1303-7.
28. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies*. *Nature*, 1997. **388**(6645): p. 839-40.
29. Benskey, M.J., et al., *Silencing Alpha Synuclein in Mature Nigral Neurons Results in Rapid Neuroinflammation and Subsequent Toxicity*. *Front Mol Neurosci*, 2018. **11**: p. 36.
30. Dauer, W., et al., *Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP*. *Proc Natl Acad Sci U S A*, 2002. **99**(22): p. 14524-9.
31. Halliday, G.M., et al., *Neuropathology of immunohistochemically identified brainstem neurons in Parkinson's disease*. *Ann Neurol*, 1990. **27**(4): p. 373-85.
32. Braak, H., et al., *Staging of brain pathology related to sporadic Parkinson's disease*. *Neurobiol Aging*, 2003. **24**(2): p. 197-211.
33. Scott, D. and S. Roy, *α -Synuclein inhibits intersynaptic vesicle mobility and maintains recycling-pool homeostasis*. *J Neurosci*, 2012. **32**(30): p. 10129-35.
34. Burré, J., et al., *Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro*. *Science*, 2010. **329**(5999): p. 1663-7.
35. Cooper, A.A., et al., *Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models*. *Science*, 2006. **313**(5785): p. 324-8.
36. Krüger, R., et al., *Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease*. *Nat Genet*, 1998. **18**(2): p. 106-8.
37. Zarranz, J.J., et al., *The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia*. *Ann Neurol*, 2004. **55**(2): p. 164-73.
38. Vekrellis, K., H.J. Rideout, and L. Stefanis, *Neurobiology of alpha-synuclein*. *Mol Neurobiol*, 2004. **30**(1): p. 1-21.
39. Anderson, J.P., et al., *Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease*. *J Biol Chem*, 2006. **281**(40): p. 29739-52.
40. Giasson, B.I., et al., *A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly*. *J Biol Chem*, 2001. **276**(4): p. 2380-6.
41. Li, W., et al., *Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutations*. *Proc Natl Acad Sci U S A*, 2005. **102**(6): p. 2162-7.
42. McGeer, P.L., et al., *Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains*. *Neurology*, 1988. **38**(8): p. 1285-91.
43. Petroi, D., et al., *Aggregate clearance of α -synuclein in *Saccharomyces cerevisiae* depends more on autophagosome and vacuole function than on the proteasome*. *J Biol Chem*, 2012. **287**(33): p. 27567-79.
44. Xilouri, M., et al., *alpha-synuclein degradation by autophagic pathways: a potential key to Parkinson's disease pathogenesis*. *Autophagy*, 2008. **4**(7): p. 917-9.
45. Cuervo, A.M., et al., *Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy*. *Science*, 2004. **305**(5688): p. 1292-5.

46. Alvarez-Erviti, L., et al., *Chaperone-mediated autophagy markers in Parkinson disease brains*. Arch Neurol, 2010. **67**(12): p. 1464-72.
47. Vogiatzi, T., et al., *Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells*. J Biol Chem, 2008. **283**(35): p. 23542-56.
48. Xilouri, M., et al., *Impairment of chaperone-mediated autophagy induces dopaminergic neurodegeneration in rats*. Autophagy, 2016. **12**(11): p. 2230-2247.
49. Langston, J.W., et al., *Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis*. Science, 1983. **219**(4587): p. 979-80.
50. Schapira, A.H., et al., *Mitochondrial complex I deficiency in Parkinson's disease*. J Neurochem, 1990. **54**(3): p. 823-7.
51. Mizuno, Y., et al., *Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease*. Biochem Biophys Res Commun, 1989. **163**(3): p. 1450-5.
52. Di Maio, R., et al., *α -Synuclein binds to TOM20 and inhibits mitochondrial protein import in Parkinson's disease*. Sci Transl Med, 2016. **8**(342): p. 342ra78.
53. Ludtmann, M.H.R., et al., *α -synuclein oligomers interact with ATP synthase and open the permeability transition pore in Parkinson's disease*. Nat Commun, 2018. **9**(1): p. 2293.
54. Gilks, W.P., et al., *A common LRRK2 mutation in idiopathic Parkinson's disease*. Lancet, 2005. **365**(9457): p. 415-6.
55. Nichols, W.C., et al., *Genetic screening for a single common LRRK2 mutation in familial Parkinson's disease*. Lancet, 2005. **365**(9457): p. 410-2.
56. Di Fonzo, A., et al., *A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease*. Lancet, 2005. **365**(9457): p. 412-5.
57. Barrett, J.C., et al., *Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease*. Nat Genet, 2008. **40**(8): p. 955-62.
58. Di Maio, R., et al., *LRRK2 activation in idiopathic Parkinson's disease*. Sci Transl Med, 2018. **10**(451).
59. Giasson, B.I., et al., *Biochemical and pathological characterization of Lrrk2*. Ann Neurol, 2006. **59**(2): p. 315-22.
60. Zhu, X., et al., *LRRK2 protein is a component of Lewy bodies*. Ann Neurol, 2006. **60**(5): p. 617-618.
61. Monfrini, E. and A. Di Fonzo, *Leucine-Rich Repeat Kinase (LRRK2) Genetics and Parkinson's Disease*, in *Leucine-Rich Repeat Kinase 2 (LRRK2)*, H.J. Rideout, Editor. 2017, Springer International Publishing: Cham. p. 3-30.
62. Gan-Or, Z., et al., *LRRK2 mutations in Parkinson disease; a sex effect or lack thereof? A meta-analysis*. Parkinsonism Relat Disord, 2015. **21**(7): p. 778-82.
63. Kestenbaum, M. and R.N. Alcalay, *Clinical Features of LRRK2 Carriers with Parkinson's Disease*, in *Leucine-Rich Repeat Kinase 2 (LRRK2)*, H.J. Rideout, Editor. 2017, Springer International Publishing: Cham. p. 31-48.
64. Clark, L.N., et al., *Frequency of LRRK2 mutations in early- and late-onset Parkinson disease*. Neurology, 2006. **67**(10): p. 1786-91.
65. Adams, J.R., et al., *PET in LRRK2 mutations: comparison to sporadic Parkinson's disease and evidence for presymptomatic compensation*. Brain, 2005. **128**(Pt 12): p. 2777-85.
66. Funayama, M., et al., *A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1*. Ann Neurol, 2002. **51**(3): p. 296-301.
67. Marín, I., W.N. van Egmond, and P.J. van Haastert, *The Roco protein family: a functional perspective*. Faseb j, 2008. **22**(9): p. 3103-10.
68. Deng, J., et al., *Structure of the ROC domain from the Parkinson's disease-associated leucine-rich repeat kinase 2 reveals a dimeric GTPase*. Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1499-504.

69. Mills, R.D., et al., *The Roc-COR tandem domain of leucine-rich repeat kinase 2 forms dimers and exhibits conventional Ras-like GTPase properties*. J Neurochem, 2018. **147**(3): p. 409-428.
70. Antoniou, N., et al., *A motif within the armadillo repeat of Parkinson's-linked LRRK2 interacts with FADD to hijack the extrinsic death pathway*. Sci Rep, 2018. **8**(1): p. 3455.
71. Vides, E.G., et al., *A feed-forward pathway drives LRRK2 kinase membrane recruitment and activation*. Elife, 2022. **11**.
72. Chia, R., et al., *Phosphorylation of LRRK2 by casein kinase 1 α regulates trans-Golgi clustering via differential interaction with ARHGEF7*. Nat Commun, 2014. **5**: p. 5827.
73. Dzamko, N., et al., *The I κ B kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during Toll-like receptor signaling*. PLoS One, 2012. **7**(6): p. e39132.
74. Li, X., et al., *Phosphorylation-dependent 14-3-3 binding to LRRK2 is impaired by common mutations of familial Parkinson's disease*. PLoS One, 2011. **6**(3): p. e17153.
75. Mosavi, L.K., et al., *The ankyrin repeat as molecular architecture for protein recognition*. Protein Sci, 2004. **13**(6): p. 1435-48.
76. Striegl, H., M.A. Andrade-Navarro, and U. Heinemann, *Armadillo motifs involved in vesicular transport*. PLoS One, 2010. **5**(2): p. e8991.
77. Ng, A. and R.J. Xavier, *Leucine-rich repeat (LRR) proteins: Integrators of pattern recognition and signaling in immunity*. Autophagy, 2011. **7**(9): p. 1082-1084.
78. Jaleel, M., et al., *LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity*. Biochem J, 2007. **405**(2): p. 307-17.
79. Piccoli, G., et al., *Leucine-rich repeat kinase 2 binds to neuronal vesicles through protein interactions mediated by its C-terminal WD40 domain*. Mol Cell Biol, 2014. **34**(12): p. 2147-61.
80. Sheng, Z., et al., *Ser1292 autophosphorylation is an indicator of LRRK2 kinase activity and contributes to the cellular effects of PD mutations*. Sci Transl Med, 2012. **4**(164): p. 164ra161.
81. Greggio, E., et al., *The Parkinson's disease kinase LRRK2 autophosphorylates its GTPase domain at multiple sites*. Biochem Biophys Res Commun, 2009. **389**(3): p. 449-54.
82. Gloeckner, C.J., et al., *Phosphopeptide analysis reveals two discrete clusters of phosphorylation in the N-terminus and the Roc domain of the Parkinson-disease associated protein kinase LRRK2*. J Proteome Res, 2010. **9**(4): p. 1738-45.
83. Healy, D.G., et al., *Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study*. Lancet Neurol, 2008. **7**(7): p. 583-90.
84. Mata, I.F., et al., *The discovery of LRRK2 p.R1441S, a novel mutation for Parkinson's disease, adds to the complexity of a mutational hotspot*. Am J Med Genet B Neuropsychiatr Genet, 2016. **171**(7): p. 925-30.
85. Mata, I.F., et al., *Lrrk2 pathogenic substitutions in Parkinson's disease*. Neurogenetics, 2005. **6**(4): p. 171-7.
86. Xiomerisiou, G., et al., *Screening for SNCA and LRRK2 mutations in Greek sporadic and autosomal dominant Parkinson's disease: identification of two novel LRRK2 variants*. Eur J Neurol, 2007. **14**(1): p. 7-11.
87. Melachroinou, K., et al., *Activation of FADD-Dependent Neuronal Death Pathways as a Predictor of Pathogenicity for LRRK2 Mutations*. PLoS One, 2016. **11**(11): p. e0166053.

88. Ozelius, L.J., et al., *LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews*. N Engl J Med, 2006. **354**(4): p. 424-5.
89. Lesage, S., et al., *LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs*. N Engl J Med, 2006. **354**(4): p. 422-3.
90. Ishihara, L., et al., *Clinical features of Parkinson disease patients with homozygous leucine-rich repeat kinase 2 G2019S mutations*. Arch Neurol, 2006. **63**(9): p. 1250-4.
91. Lai, D., et al., *Genomewide Association Studies of LRRK2 Modifiers of Parkinson's Disease*. Ann Neurol, 2021. **90**(1): p. 76-88.
92. Botta-Orfila, T., et al., *Age at onset in LRRK2-associated PD is modified by SNCA variants*. J Mol Neurosci, 2012. **48**(1): p. 245-7.
93. Trinh, J., et al., *The role of SNCA and MAPT in Parkinson disease and LRRK2 parkinsonism in the Tunisian Arab-Berber population*. Eur J Neurol, 2014. **21**(11): p. e91-2.
94. Ross, O.A., et al., *Analysis of Lrrk2 R1628P as a risk factor for Parkinson's disease*. Ann Neurol, 2008. **64**(1): p. 88-92.
95. Leandrou, E., et al., *Kinase activity of mutant LRRK2 manifests differently in hetero-dimeric vs. homo-dimeric complexes*. Biochem J, 2019. **476**(3): p. 559-579.
96. West, A.B., et al., *Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity*. Hum Mol Genet, 2007. **16**(2): p. 223-32.
97. Nichols, R.J., et al., *14-3-3 binding to LRRK2 is disrupted by multiple Parkinson's disease-associated mutations and regulates cytoplasmic localization*. Biochem J, 2010. **430**(3): p. 393-404.
98. Greggio, E., et al., *Kinase activity is required for the toxic effects of mutant LRRK2/dardarin*. Neurobiol Dis, 2006. **23**(2): p. 329-41.
99. Steger, M., et al., *Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a subset of Rab GTPases*. Elife, 2016. **5**.
100. Sen, S., P.J. Webber, and A.B. West, *Dependence of leucine-rich repeat kinase 2 (LRRK2) kinase activity on dimerization*. J Biol Chem, 2009. **284**(52): p. 36346-36356.
101. Biosa, A., et al., *GTPase activity regulates kinase activity and cellular phenotypes of Parkinson's disease-associated LRRK2*. Hum Mol Genet, 2013. **22**(6): p. 1140-56.
102. Lewis, P.A., et al., *The R1441C mutation of LRRK2 disrupts GTP hydrolysis*. Biochem Biophys Res Commun, 2007. **357**(3): p. 668-71.
103. Smith, W.W., et al., *Kinase activity of mutant LRRK2 mediates neuronal toxicity*. Nat Neurosci, 2006. **9**(10): p. 1231-3.
104. Nguyen, A.P.T., et al., *Dopaminergic neurodegeneration induced by Parkinson's disease-linked G2019S LRRK2 is dependent on kinase and GTPase activity*. Proc Natl Acad Sci U S A, 2020. **117**(29): p. 17296-17307.
105. West, A.B., et al., *Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity*. Proc Natl Acad Sci U S A, 2005. **102**(46): p. 16842-7.
106. Reynolds, A., et al., *LRRK2 kinase activity and biology are not uniformly predicted by its autophosphorylation and cellular phosphorylation site status*. Front Mol Neurosci, 2014. **7**: p. 54.
107. Greggio, E., et al., *The Parkinson disease-associated leucine-rich repeat kinase 2 (LRRK2) is a dimer that undergoes intramolecular autophosphorylation*. J Biol Chem, 2008. **283**(24): p. 16906-14.
108. Berger, Z., K.A. Smith, and M.J. Lavoie, *Membrane localization of LRRK2 is associated with increased formation of the highly active LRRK2 dimer and changes in its phosphorylation*. Biochemistry, 2010. **49**(26): p. 5511-23.

109. Ito, G., et al., *Lack of correlation between the kinase activity of LRRK2 harboring kinase-modifying mutations and its phosphorylation at Ser910, 935, and Ser955*. PLoS One, 2014. **9**(5): p. e97988.
110. Chia, R., et al., *Phosphorylation of LRRK2 by casein kinase 1 α regulates trans-Golgi clustering via differential interaction with ARHGEF7*. Nature Communications, 2014. **5**(1): p. 5827.
111. Manschwetus, J.T., et al., *Binding of the Human 14-3-3 Isoforms to Distinct Sites in the Leucine-Rich Repeat Kinase 2*. Front Neurosci, 2020. **14**: p. 302.
112. Hatano, T., et al., *Leucine-rich repeat kinase 2 associates with lipid rafts*. Human Molecular Genetics, 2007. **16**(6): p. 678-690.
113. Biskup, S., et al., *Localization of LRRK2 to membranous and vesicular structures in mammalian brain*. Ann Neurol, 2006. **60**(5): p. 557-569.
114. Dzamko, N., et al., *Inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser(910)/Ser(935), disruption of 14-3-3 binding and altered cytoplasmic localization*. Biochem J, 2010. **430**(3): p. 405-13.
115. Mamais, A., et al., *Arsenite stress down-regulates phosphorylation and 14-3-3 binding of leucine-rich repeat kinase 2 (LRRK2), promoting self-association and cellular redistribution*. J Biol Chem, 2014. **289**(31): p. 21386-400.
116. Lavalley, N.J., et al., *14-3-3 Proteins regulate mutant LRRK2 kinase activity and neurite shortening*. Hum Mol Genet, 2016. **25**(1): p. 109-22.
117. Wang, X., et al., *Rab12 is a regulator of LRRK2 and its activation by damaged lysosomes*. Elife, 2023. **12**.
118. Dhekne, H.S., et al., *Genome-wide screen reveals Rab12 GTPase as a critical activator of pathogenic LRRK2 kinase*. bioRxiv, 2023: p. 2023.02.17.529028.
119. Zhu, H., et al., *Rab29-dependent asymmetrical activation of leucine-rich repeat kinase 2*. Science, 2023. **382**(6677): p. 1404-1411.
120. Lobbstaël, E., et al., *Identification of protein phosphatase 1 as a regulator of the LRRK2 phosphorylation cycle*. Biochem J, 2013. **456**(1): p. 119-28.
121. Athanasopoulos, P.S., et al., *Identification of protein phosphatase 2A as an interacting protein of leucine-rich repeat kinase 2*. Biol Chem, 2016. **397**(6): p. 541-54.
122. Berndsen, K., et al., *PPM1H phosphatase counteracts LRRK2 signaling by selectively dephosphorylating Rab proteins*. Elife, 2019. **8**.
123. Liu, W., et al., *LRRK2 promotes the activation of NLRC4 inflammasome during Salmonella Typhimurium infection*. J Exp Med, 2017. **214**(10): p. 3051-3066.
124. Kalogeropoulou, A.F., et al., *P62/SQSTM1 is a novel leucine-rich repeat kinase 2 (LRRK2) substrate that enhances neuronal toxicity*. Biochem J, 2018. **475**(7): p. 1271-1293.
125. Miklossy, J., et al., *LRRK2 expression in normal and pathologic human brain and in human cell lines*. J Neuropathol Exp Neurol, 2006. **65**(10): p. 953-63.
126. Taymans, J.M., C. Van den Haute, and V. Baekelandt, *Distribution of PINK1 and LRRK2 in rat and mouse brain*. J Neurochem, 2006. **98**(3): p. 951-61.
127. Higashi, S., et al., *Expression and localization of Parkinson's disease-associated leucine-rich repeat kinase 2 in the mouse brain*. J Neurochem, 2007. **100**(2): p. 368-81.
128. West, A.B., et al., *Differential LRRK2 expression in the cortex, striatum, and substantia nigra in transgenic and nontransgenic rodents*. J Comp Neurol, 2014. **522**(11): p. 2465-80.
129. Fan, Y., et al., *Interrogating Parkinson's disease LRRK2 kinase pathway activity by assessing Rab10 phosphorylation in human neutrophils*. Biochem J, 2018. **475**(1): p. 23-44.
130. Kim, B., et al., *Impaired inflammatory responses in murine Lrrk2-knockdown brain microglia*. PLoS One, 2012. **7**(4): p. e34693.

131. Russo, I., et al., *Erratum to: Leucine-rich repeat kinase 2 positively regulates inflammation and down-regulates NF- κ B p50 signaling in cultured microglia cells*. J Neuroinflammation, 2016. **13**(1): p. 70.
132. Moehle, M.S., et al., *LRRK2 inhibition attenuates microglial inflammatory responses*. J Neurosci, 2012. **32**(5): p. 1602-11.
133. Panagiotakopoulou, V., et al., *Interferon- γ signaling synergizes with LRRK2 in neurons and microglia derived from human induced pluripotent stem cells*. Nature Communications, 2020. **11**(1): p. 5163.
134. Cook, D.A., et al., *LRRK2 levels in immune cells are increased in Parkinson's disease*. NPJ Parkinsons Dis, 2017. **3**: p. 11.
135. Orenstein, S.J., et al., *Interplay of LRRK2 with chaperone-mediated autophagy*. Nature Neuroscience, 2013. **16**(4): p. 394-406.
136. Manzoni, C., et al., *Pathogenic Parkinson's disease mutations across the functional domains of LRRK2 alter the autophagic/lysosomal response to starvation*. Biochemical and Biophysical Research Communications, 2013. **441**(4): p. 862-866.
137. Schapansky, J., et al., *Familial knockin mutation of LRRK2 causes lysosomal dysfunction and accumulation of endogenous insoluble α -synuclein in neurons*. Neurobiology of Disease, 2018. **111**: p. 26-35.
138. Bravo-San Pedro, J.M., et al., *The LRRK2 G2019S mutant exacerbates basal autophagy through activation of the MEK/ERK pathway*. Cellular and Molecular Life Sciences, 2013. **70**(1): p. 121-136.
139. Ramonet, D., et al., *Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2*. PLoS One, 2011. **6**(4): p. e18568.
140. Alegre-Abarrategui, J., et al., *LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model*. Hum Mol Genet, 2009. **18**(21): p. 4022-34.
141. Ho, P.W.-L., et al., *Age-dependent accumulation of oligomeric SNCA/ α -synuclein from impaired degradation in mutant LRRK2 knockin mouse model of Parkinson disease: role for therapeutic activation of chaperone-mediated autophagy (CMA)*. Autophagy, 2020. **16**(2): p. 347-370.
142. Schapansky, J., et al., *Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy*. Hum Mol Genet, 2014. **23**(16): p. 4201-14.
143. Tong, Y., et al., *Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice*. Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9879-84.
144. Ho, C.C., et al., *The Parkinson disease protein leucine-rich repeat kinase 2 transduces death signals via Fas-associated protein with death domain and caspase-8 in a cellular model of neurodegeneration*. J Neurosci, 2009. **29**(4): p. 1011-6.
145. Niu, J., et al., *Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via Dynamin-like protein*. J Neurochem, 2012. **122**(3): p. 650-8.
146. Williamson, M.G., et al., *Mitochondrial dysfunction and mitophagy defects in LRRK2-R1441C Parkinson's disease models*. Hum Mol Genet, 2023. **32**(18): p. 2808-2821.
147. Singh, F., et al., *Pharmacological rescue of impaired mitophagy in Parkinson's disease-related LRRK2 G2019S knock-in mice*. Elife, 2021. **10**.
148. Lin, X., et al., *Leucine-rich repeat kinase 2 regulates the progression of neuropathology induced by Parkinson's-disease-related mutant alpha-synuclein*. Neuron, 2009. **64**(6): p. 807-27.
149. Herzig, M.C., et al., *High LRRK2 levels fail to induce or exacerbate neuronal alpha-synucleinopathy in mouse brain*. PLoS One, 2012. **7**(5): p. e36581.

150. Emmanouilidou, E., et al., *Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival*. J Neurosci, 2010. **30**(20): p. 6838-51.
151. Daher, J.P., et al., *Leucine-rich Repeat Kinase 2 (LRRK2) Pharmacological Inhibition Abates α -Synuclein Gene-induced Neurodegeneration*. J Biol Chem, 2015. **290**(32): p. 19433-44.
152. Longo, F., et al., *Age-dependent dopamine transporter dysfunction and Serine129 phospho- α -synuclein overload in G2019S LRRK2 mice*. Acta Neuropathol Commun, 2017. **5**(1): p. 22.
153. Volpicelli-Daley, L.A., et al., *G2019S-LRRK2 Expression Augments α -Synuclein Sequestration into Inclusions in Neurons*. J Neurosci, 2016. **36**(28): p. 7415-27.
154. Schapansky, J., et al., *Familial knockin mutation of LRRK2 causes lysosomal dysfunction and accumulation of endogenous insoluble α -synuclein in neurons*. Neurobiol Dis, 2018. **111**: p. 26-35.
155. Daher, J.P., et al., *Abrogation of α -synuclein-mediated dopaminergic neurodegeneration in LRRK2-deficient rats*. Proc Natl Acad Sci U S A, 2014. **111**(25): p. 9289-94.
156. Henderson, J.L., et al., *Discovery and preclinical profiling of 3-[4-(morpholin-4-yl)-7H-pyrrolo[2,3-d]pyrimidin-5-yl]benzonitrile (PF-06447475), a highly potent, selective, brain penetrant, and in vivo active LRRK2 kinase inhibitor*. J Med Chem, 2015. **58**(1): p. 419-32.
157. Bieri, G., et al., *LRRK2 modifies α -syn pathology and spread in mouse models and human neurons*. Acta Neuropathol, 2019. **137**(6): p. 961-980.
158. Henderson, M.X., et al., *LRRK2 inhibition does not impart protection from α -synuclein pathology and neuron death in non-transgenic mice*. Acta Neuropathol Commun, 2019. **7**(1): p. 28.
159. Stenmark, H., *Rab GTPases as coordinators of vesicle traffic*. Nature Reviews Molecular Cell Biology, 2009. **10**(8): p. 513-525.
160. Jeong, G.R., et al., *Dysregulated phosphorylation of Rab GTPases by LRRK2 induces neurodegeneration*. Mol Neurodegener, 2018. **13**(1): p. 8.
161. Ito, G., et al., *Phos-tag analysis of Rab10 phosphorylation by LRRK2: a powerful assay for assessing kinase function and inhibitors*. Biochemical Journal, 2016. **473**(17): p. 2671-2685.
162. McGrath, E., et al., *LRRK2 binds to the Rab32 subfamily in a GTP-dependent manner via its armadillo domain*. Small GTPases, 2021. **12**(2): p. 133-146.
163. Dhekne, H.S., et al., *A pathway for Parkinson's Disease LRRK2 kinase to block primary cilia and Sonic hedgehog signaling in the brain*. Elife, 2018. **7**.
164. Lara Ordóñez, A.J., et al., *RAB8, RAB10 and RILPL1 contribute to both LRRK2 kinase-mediated centrosomal cohesion and ciliogenesis deficits*. Hum Mol Genet, 2019. **28**(21): p. 3552-3568.
165. Rivero-Ríos, P., et al., *The G2019S variant of leucine-rich repeat kinase 2 (LRRK2) alters endolysosomal trafficking by impairing the function of the GTPase RAB8A*. J Biol Chem, 2019. **294**(13): p. 4738-4758.
166. MacLeod, D.A., et al., *RAB7L1 interacts with LRRK2 to modify intraneuronal protein sorting and Parkinson's disease risk*. Neuron, 2013. **77**(3): p. 425-39.
167. Kalogeropoulou, A.F., et al., *Endogenous Rab29 does not impact basal or stimulated LRRK2 pathway activity*. Biochem J, 2020. **477**(22): p. 4397-4423.
168. Kluss, J.H., et al., *Directing LRRK2 to membranes of the endolysosomal pathway triggers RAB phosphorylation and JIP4 recruitment*. Neurobiol Dis, 2022. **170**: p. 105769.
169. Bondar, V.V., et al., *Rab12 regulates LRRK2 activity by promoting its localization to lysosomes*. bioRxiv, 2023: p. 2023.02.21.529466.

170. Chiu, C.C., et al., *Increased Rab35 expression is a potential biomarker and implicated in the pathogenesis of Parkinson's disease*. *Oncotarget*, 2016. **7**(34): p. 54215-54227.
171. Gitler, A.D., et al., *The Parkinson's disease protein alpha-synuclein disrupts cellular Rab homeostasis*. *Proc Natl Acad Sci U S A*, 2008. **105**(1): p. 145-50.
172. *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework*. *Clin Pharmacol Ther*, 2001. **69**(3): p. 89-95.
173. Dzamko, N., et al., *Measurement of LRRK2 and Ser910/935 phosphorylated LRRK2 in peripheral blood mononuclear cells from idiopathic Parkinson's disease patients*. *J Parkinsons Dis*, 2013. **3**(2): p. 145-52.
174. Melachroinou, K., et al., *Elevated In Vitro Kinase Activity in Peripheral Blood Mononuclear Cells of Leucine-Rich Repeat Kinase 2 G2019S Carriers: A Novel Enzyme-Linked Immunosorbent Assay-Based Method*. *Mov Disord*, 2020. **35**(11): p. 2095-2100.
175. Padmanabhan, S., et al., *An Assessment of LRRK2 Serine 935 Phosphorylation in Human Peripheral Blood Mononuclear Cells in Idiopathic Parkinson's Disease and G2019S LRRK2 Cohorts*. *J Parkinsons Dis*, 2020. **10**(2): p. 623-629.
176. Iannotta, L., et al., *Divergent Effects of G2019S and R1441C LRRK2 Mutations on LRRK2 and Rab10 Phosphorylations in Mouse Tissues*. *Cells*, 2020. **9**(11).
177. Perera, G., et al., *Inhibitor treatment of peripheral mononuclear cells from Parkinson's disease patients further validates LRRK2 dephosphorylation as a pharmacodynamic biomarker*. *Sci Rep*, 2016. **6**: p. 31391.
178. Reith, A.D., et al., *GSK2578215A; A potent and highly selective 2-arylmethoxy-5-substituent-N-arylbenzamide LRRK2 kinase inhibitor*. *Bioorganic & Medicinal Chemistry Letters*, 2012. **22**(17): p. 5625-5629.
179. Atashrazm, F., et al., *LRRK2-mediated Rab10 phosphorylation in immune cells from Parkinson's disease patients*. *Mov Disord*, 2019. **34**(3): p. 406-415.
180. Fan, Y., et al., *R1441G but not G2019S mutation enhances LRRK2 mediated Rab10 phosphorylation in human peripheral blood neutrophils*. *Acta Neuropathol*, 2021. **142**(3): p. 475-494.
181. Wang, X., et al., *Understanding LRRK2 kinase activity in preclinical models and human subjects through quantitative analysis of LRRK2 and pT73 Rab10*. *Sci Rep*, 2021. **11**(1): p. 12900.
182. Fraser, K.B., et al., *LRRK2 secretion in exosomes is regulated by 14-3-3*. *Hum Mol Genet*, 2013. **22**(24): p. 4988-5000.
183. Fraser, K.B., et al., *Ser(P)-1292 LRRK2 in urinary exosomes is elevated in idiopathic Parkinson's disease*. *Mov Disord*, 2016. **31**(10): p. 1543-1550.
184. Wang, S., et al., *Elevated LRRK2 autophosphorylation in brain-derived and peripheral exosomes in LRRK2 mutation carriers*. *Acta Neuropathol Commun*, 2017. **5**(1): p. 86.
185. Fraser, K.B., et al., *Urinary LRRK2 phosphorylation predicts parkinsonian phenotypes in G2019S LRRK2 carriers*. *Neurology*, 2016. **86**(11): p. 994-9.
186. Wang, S., et al., *Exosome markers of LRRK2 kinase inhibition*. *NPJ Parkinsons Dis*, 2020. **6**(1): p. 32.
187. Mabrouk, O.S., et al., *Quantitative Measurements of LRRK2 in Human Cerebrospinal Fluid Demonstrates Increased Levels in G2019S Patients*. *Front Neurosci*, 2020. **14**: p. 526.
188. Ho, D.H., et al., *Increased DJ-1 in urine exosome of Korean males with Parkinson's disease*. *Biomed Res Int*, 2014. **2014**: p. 704678.
189. Nam, D., et al., *Analysis of α -synuclein levels related to LRRK2 kinase activity: from substantia nigra to urine of patients with Parkinson's disease*. *Anim Cells Syst (Seoul)*, 2021. **25**(1): p. 28-36.
190. Shi, M., et al., *Plasma exosomal α -synuclein is likely CNS-derived and increased in Parkinson's disease*. *Acta Neuropathol*, 2014. **128**(5): p. 639-650.

191. Goetzl, E.J., et al., *Altered lysosomal proteins in neural-derived plasma exosomes in preclinical Alzheimer disease*. Neurology, 2015. **85**(1): p. 40-7.
192. Mustapic, M., et al., *Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes*. Front Neurosci, 2017. **11**: p. 278.
193. Jiang, C., et al., *Validation of α -Synuclein in L1CAM-Immunocaptured Exosomes as a Biomarker for the Stratification of Parkinsonian Syndromes*. Mov Disord, 2021. **36**(11): p. 2663-2669.
194. Rikkert, L.G., et al., *Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent*. J Extracell Vesicles, 2019. **8**(1): p. 1555419.
195. Goetzl, E.J., et al., *Decreased synaptic proteins in neuronal exosomes of frontotemporal dementia and Alzheimer's disease*. Faseb j, 2016. **30**(12): p. 4141-4148.
196. Goetzl, E.J., et al., *Cargo proteins of plasma astrocyte-derived exosomes in Alzheimer's disease*. Faseb j, 2016. **30**(11): p. 3853-3859.
197. Kluss, J.H., et al., *Detection of endogenous S1292 LRRK2 autophosphorylation in mouse tissue as a readout for kinase activity*. NPJ Parkinsons Dis, 2018. **4**: p. 13.
198. Wang, S., et al., *Elevated Urinary Rab10 Phosphorylation in Idiopathic Parkinson Disease*. Mov Disord, 2022. **37**(7): p. 1454-1464.

APPENDICES

I. Πληροφοριακό Σημείωμα για τη συμμετοχή στη μελέτη: «Βιοδείκτες βασισμένοι στην πρωτεΐνη LRRK2 στη νόσο Πάρκινσον»

Αγαπητέ/Αγαπητή συμμετέχοντα στην μελέτη,

Οι πληροφορίες που περιέχονται σε αυτό το σημείωμα στοχεύουν στην ενημέρωσή σας σχετικά με τους στόχους, την πορεία, τα πλεονεκτήματα, αλλά και πιθανά μειονεκτήματα που ενδέχεται να παρουσιαστούν κατά την διάρκεια της Μελέτης. Αυτό το Ενημερωτικό Σημείωμα μπορεί να περιέχει εκφράσεις που δεν γνωρίζετε ή δεν καταλαβαίνετε. Παρακαλώ διαβάστε το προσεκτικά και απευθύνετε όσες ερωτήσεις πιστεύετε είναι απαραίτητες. Παρακαλώ μην υπογράψετε για την συμμετοχή σας, παρά μόνον εάν όλα τα ερωτήματά σας απαντηθούν με ικανοποιητικό τρόπο. Παρακαλώ επικοινωνήστε με ένα από τα μέλη της Ερευνητικής ομάδας ή με τον Ιατρό της Μελέτης στην περίπτωση που υπάρχουν ερωτήσεις, ή ορισμένες από τις έννοιες δεν είναι κατανοητές. Σε περίπτωση που θέλετε να συζητήσετε την συμμετοχή σας στην Μελέτη με άλλο ή άλλα πρόσωπα, είναι δυνατόν να σας δοθεί ένα αντίγραφο του Πληροφοριακού Σημειώματος πριν υπογράψετε.

Εισαγωγή

Η νόσος Πάρκινσον είναι μία νευρολογική διαταραχή. Πρόκειται για την δεύτερη συνηθέστερη νευροεκφυλιστική διαταραχή μετά τη νόσο Αλτσχάιμερ. Επηρεάζει περίπου 1% του πληθυσμού άνω των 65 ετών. Η μέση ηλικία έναρξης είναι τα 60 έτη. Αφορά στα νευρικά κύτταρα μιας συγκεκριμένης περιοχής του εγκεφάλου που ονομάζεται μέλαινα ουσία, τα οποία παθαίνουν βλάβη ή πεθαίνουν. Φυσιολογικά, τα κύτταρα αυτά παράγουν μία ουσία η οποία ονομάζεται ντοπαμίνη και επιτρέπει την ομαλή και συντονισμένη κίνηση των μυών. Όταν μεγάλο ποσοστό των κυττάρων αυτών υποστούν βλάβη, εμφανίζονται τα συμπτώματα της νόσου δηλαδή ο τρόμος, η βραδύτητα των κινήσεων, η δυσκαμψία και η διαταραχή της ισορροπίας.

Μέχρι τώρα η αιτία της νόσου Πάρκινσον είναι άγνωστη. Μελέτες που έχουν γίνει συγκλίνουν στη διαπίστωση ότι σε πολλές περιπτώσεις, προκαλείται είτε από γενετικές ανωμαλίες είτε από την επίδραση περιβαλλοντικών παραγόντων σε γενετικά προδιατεθειμένα άτομα. Έχει διαπιστωθεί τελευταία ότι υπάρχει οικογενειακό ιστορικό της νόσου σε ποσοστό 5-10% των ασθενών. Έχουν ενοχοποιηθεί συγκεκριμένα γονίδια για πρόκληση της νόσου σε οικογένειες με νόσο Πάρκινσον. Μελέτες σε

οικογένειες ασθενών μπορούν να οδηγήσουν σε περαιτέρω διαλεύκανση της αιτιολογίας της νόσου. Έχουν μέχρι στιγμής ανευρεθεί 15 γονιδιακοί τόποι, δηλαδή περιοχές του γενετικού υλικού όπου υπάρχουν γενετικές παραλλαγές που σχετίζονται με την ανάπτυξη της νόσου. Ανάμεσα σε αυτά τα γονίδια, το γονίδιο SNCA, που κωδικοποιεί για την πρωτεΐνη α-συνουκλεΐνη είναι ιδιαίτερα σημαντικό. Η ανώμαλη εναπόθεση α-συνουκλεΐνης αποτελεί βασικό παθολογοανατομικό χαρακτηριστικό της νόσου.

Στην παρούσα μελέτη διερευνούμε αν μπορούμε να ανιχνεύσουμε αλλαγές στη δραστηριότητα μιας πρωτεΐνης που ονομάζεται LRRK2 σε βιολογικά δείγματα από άτομα που έχουν προσβληθεί από νόσο Πάρκινσον και που είτε έχουν την κλασσική ιδιοπαθή μορφή της νόσου, είτε είναι φορείς μιας μετάλλαξης (A53T) στο γονίδιο της άλφα-συνουκλεΐνης. Γιατί να μελετήσουμε τη δραστηριότητα της LRRK2 σε αυτούς τους ασθενείς; Πρώτον, υπάρχουν τώρα σημαντικά στοιχεία ότι αυτή η πρωτεΐνη εμπλέκεται στον τύπο της φλεγμονώδους απόκρισης που παρατηρείται συχνά στους ασθενείς με νόσο Πάρκινσον. Φαίνεται ότι η απώλεια της δραστηριότητας της πρωτεΐνης αυτής βελτιώνει την επιβίωση των νευρώνων της, μεταβάλλοντας την παθολογική συσσώρευση της άλφα-συνουκλεΐνης. Δεύτερον, η LRRK2 είναι επίσης πολύ σημαντική σε ζωικά μοντέλα με νόσο Πάρκινσον. Σε αυτή τη μελέτη, θα διερευνήσουμε εάν τα επίπεδα ή η δραστηριότητα της LRRK2 μεταβάλλονται σε πολλαπλά δείγματα βιολογικών υλικών σε ασθενείς με νόσο Πάρκινσον, με ειδικό ενδιαφέρον σε αυτούς που φέρουν τη συγκεκριμένη μετάλλαξη (A53T) της άλφα-συνουκλεΐνης. Για τους ερευνητικούς σκοπούς μας θα χρησιμοποιήσουμε 2 βιολογικά υλικά, αίμα και ούρα τα οποία θα επεξεργαστούμε περαιτέρω. Σκοπός της μελέτης είναι να προσδιοριστεί εάν η πρωτεΐνη αυτή (LRRK2) μπορεί να χρησιμοποιηθεί ως αξιόπιστος δείκτης για την εξέλιξη της νόσου. Τέλος, η μελέτη αυτή μπορεί να βοηθήσει επίσης σε κλινικές δοκιμές νέων φαρμακευτικών θεραπειών για τη νόσο Πάρκινσον.

Δεν είναι γνωστό ποιες είναι οι αλλαγές που συμβαίνουν στους εγκεφάλους των ασθενών πριν ακόμη νοσήσουν έκδηλα και που οδηγούν με τα χρόνια στο νευρωνικό εκφυλισμό. Είναι πολύ σημαντικό να βρεθούν βιολογικοί δείκτες που με έγκυρο τρόπο θα προβλέπουν ότι το άτομο θα νοσήσει ακόμη και πολλά χρόνια πριν την εκδήλωση της νόσου. Οι ίδιοι βιολογικοί δείκτες μπορούν να χρησιμοποιηθούν και για να παρακολουθηθεί με αντικειμενικό τρόπο η πορεία της νόσου. Αυτό θα έχει ιδιαίτερη σημασία καθώς ελπίζουμε ότι τα επόμενα χρόνια θα αναπτυχθούν θεραπείες που θα είναι νευροπροστατευτικές, θα αντιμετωπίζουν δηλαδή τη νόσο στην βάση της, και θα μπορούν να εφαρμόζονται ακόμη και σε άτομα που ακόμη δεν έχουν συμπτώματα.

Σας ζητάμε σήμερα να μας βοηθήσετε σε αυτή την προσπάθεια.

Η μελέτη αυτή πραγματοποιείται στη Νευρολογική Κλινική του Νοσοκομείου Ερρίκος Ντυνάν και στο Ίδρυμα Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών (ΙΙΒΕΑΑ) σε συνεργασία με την Α' Νευρολογική Κλινική του ΕΚΠΑ στο Αιγινήτειο Νοσοκομείο. Η διάρκειά της είναι δύο χρόνια.

Χρηματοδοτείται από δύο διεθνείς οργανισμούς:

- Parkinson's Foundation (PF); Clinical Research Award (ID# PF-CRA-1897)
- Michael J Fox Foundation for Parkinson's Research (MJFF); Research Grant (ID# MJFF 16821)

Συμμετοχή

Αφού ενημερωθείτε σχετικά με την Μελέτη και τις εξετάσεις που θα διενεργηθούν στα πλαίσιά της, θα σας ζητηθεί να υπογράψετε μία φόρμα συγκατάθεσης. Με την υπογραφή σας συμφωνείτε να λάβετε μέρος στην παρούσα Μελέτη. Δεν έχετε υποχρέωση να λάβετε μέρος στην Μελέτη. Η συμμετοχή σας είναι προαιρετική και είστε ελεύθεροι να την ακυρώσετε σε οποιοδήποτε χρονικό σημείο. Σε περίπτωση που δεν θέλετε να συμμετάσχετε στην Μελέτη, αυτό δεν θα επηρεάσει την ιατρική σας περίθαλψη. Ο Ιατρός της μελέτης, όμως, ή ο κύριος ερευνητής έχει και αυτός το δικαίωμα να τερματίσει τη συμμετοχή σας στην Μελέτη για οποιονδήποτε λόγο και σε οποιαδήποτε στιγμή.

Συμμετέχοντες στη μελέτη και συλλογή δειγμάτων

Συμμετέχετε στην Μελέτη αυτή γιατί έχετε διαγνωσθεί με νόσο Πάρκινσον. Εναλλακτικά, συμμετέχετε ως άτομο ελέγχου (με ή χωρίς άλλο νευρολογικό νόσημα). Οι ασθενείς που παρακολουθούνται στη Νευρολογική Κλινική του Νοσοκομείου Ερρίκος Ντυνάν στην Αθήνα ή στην Α' Νευρολογική Κλινική του ΕΚΠΑ, είναι πιθανοί συμμετέχοντες στη Μελέτη. Στη Μελέτη περιλαμβάνονται τρεις επισκέψεις. Κατά την πρώτη επίσκεψη κάθε συμμετέχων θα ενημερωθεί για τη Μελέτη και θα ζητηθεί να υπογράψει τα έντυπα συγκατάθεσης. Κάθε επίσκεψη από τις τρεις θα περιλαμβάνει τη συλλογή δειγμάτων αίματος καθώς και ούρων μαζί με κλινική αξιολόγηση. Τα κριτήρια αποκλεισμού συμμετοχής στη Μελέτη για τις ομάδες ασθενών περιλαμβάνουν τη συνύπαρξη/διάγνωση άλλων νευροεκφυλιστικών ασθενειών όπως η προοδευτική υπερπυρηνική παράλυση, η ατροφία πολλαπλών συστημάτων, η νόσος Huntington, η φλοιοβασική εκφύλιση ή αυτοάνοσες διαταραχές που αφορούν το ΚΝΣ. Τα κριτήρια

ένταξης για την ομάδα ελέγχου περιλαμβάνουν την έλλειψη πρόσφατου ιστορικού ουρολοίμωξης ή άλλων λοιμώξεων που απαιτούν θεραπεία με αντιβιοτικά, απουσία διάγνωσης PD σε πρώτου βαθμού συγγενή ή άλλης νευροεκφυλιστικής ή αυτοάνοσης νόσου. Ως επιπλέον ομάδα ελέγχου, θα συλλεχθούν δείγματα από ασθενείς που λαμβάνουν θεραπεία στην κλινική για άλλες νευρολογικές καταστάσεις που δεν έχουν σχέση με τη νόσο Πάρκινσον, όπως ημικρανία ή πολλαπλή σκλήρυνση.

Διαδικασία: Δειγματοληψία και Αποθήκευση των δειγμάτων

Αρχικά, μια κλινική εξέταση, επικεντρωμένη στην εξέταση κινητικών λειτουργιών, και ερωτήσεις στις οποίες θα υποβληθείτε θα επιβεβαιώσουν κατά πόσον είστε κατάλληλος/η για την συμμετοχή σας σε αυτή την μελέτη. Στην περίπτωση που η πρώτη αυτή εκτίμηση δείξει ότι είστε κατάλληλος/η για συμμετοχή στην Μελέτη, και έχετε δώσει την συγκατάθεσή σας, θα λάβουμε ένα δείγμα αίματος (4 φιαλίδια των 8 ml) και ένα δείγμα ούρων περίπου 180 ml, για την ανάλυση γενετικών πληροφοριών και βιολογικών δεικτών. Όλα τα δείγματα θα κωδικοποιούνται κατά τη στιγμή της συλλογής και τα δεδομένα ασθενούς / δείγματος θα αποθηκεύονται στην κλινική, έτσι ώστε να μην είναι διαθέσιμες πληροφορίες ταυτοποίησης σχετικά με το δείγμα στους ερευνητές που πραγματοποιούν τις απομονώσεις και τις δοκιμασίες. Τα δεδομένα σας θα χρησιμοποιηθούν μόνο για ερευνητικούς σκοπούς. Οι εξετάσεις, ωστόσο, αυτές που θα πραγματοποιηθούν στα πλαίσια της παρούσας Μελέτης δεν πρόκειται να αλλάξουν την διάγνωσή σας, ούτε την θεραπευτική σας αντιμετώπιση.

Συγκεκριμένα, στα πλαίσια της μελέτης θα διενεργηθούν τα κάτωθι :

1. Δειγματοληψία Αίματος: Θα σας ζητηθεί να υπογράψετε και ξεχωριστά κατά πόσον συγκατατίθεστε για την λήψη αίματος. Το ποσό του αίματος (περίπου 32ml) που λαμβάνεται δεν αντιπροσωπεύει απειλή για σας υγεία σας. Χρησιμοποιείται η παρακάτω διαδικασία: γίνεται καθαρισμός και αποστείρωση συγκεκριμένης περιοχής του δέρματος, που συνήθως είναι στον βραχίονα, και εισάγεται ένας φλεβοκαθετήρας δια μέσου του οποίου αναρροφάται αίμα. Το αίμα στη συνέχεια τοποθετείται σε ειδικό φιαλίδιο. Μετά την λήψη της απαιτούμενης ποσότητας, αφαιρείται ο φλεβοκαθετήρας και τοποθετείται μικρό έμπλαστρο. Μπορεί ορισμένοι ασθενείς να εμφανίσουν έναν τοπικό πόνο, ένα τοπικό αιμάτωμα, ζάλη ή λιποθυμία κατά τη διάρκεια ή μετά την αιμοληψία. Θρόμβοι ή λοιμώξεις σπάνια παρουσιάζονται.

Τα δείγματα αυτά θα αποθηκευτούν κατ' αρχήν στο τοπικό Κέντρο, και θα αναλυθούν από τα μέλη της ερευνητικής ομάδας της Μελέτης. Μέρος των εν λόγω δειγμάτων καθώς και δημογραφικά και κλινικά δεδομένα θα αποθηκευτούν σε μία κοινή Βιοτράπεζα των συμμετεχόντων Ιδρυμάτων για τουλάχιστον 20 χρόνια. Αν κατά τη διάρκεια αυτή ανακαλυφθούν νέα γονίδια ή καινούργια μονοπάτια μεταβολισμού, που ενδεχομένως συνδέονται με τη νόσο Πάρκινσον, τα δείγματά σας μπορεί να χρησιμοποιηθούν για περαιτέρω ανάλυση. Είστε, φυσικά, ο/η κάτοχος του δείγματος κατά τη διάρκεια και μετά την Μελέτη, κάτι που σας παρέχει το δικαίωμα για καταστροφή των δειγμάτων ανά πάσα στιγμή. Στην περίπτωση που επιθυμείτε κάτι τέτοιο, πρέπει να επικοινωνήσετε με τον ιατρό της Μελέτης και να τον ενημερώσετε για την απόφασή σας.

2. Κλινική αξιολόγηση. Σε κάθε επίσκεψη πραγματοποιείται κλινική αξιολόγηση. Ένας νευρολόγος στη Νευρολογική Κλινική του Νοσοκομείου Ερρίκος Ντυνάν ή της Νευρολογικής κλινικής του Αιγινήτειου Νοσοκομείου, θα πραγματοποιήσει την κλινική σας αξιολόγηση. Θα συλλεχθούν δημογραφικές πληροφορίες, πρόσφατο ιατρικό ιστορικό, το ιστορικό της νόσου (εάν είστε στην ομάδα ασθενών) και ιστορικό φαρμάκων. Η εκτίμηση της κινητικότητας θα πραγματοποιηθεί με την τροποποιημένη κλίμακα αξιολόγησης της νόσου του Parkinson (MDS-UPDRS, 4 ενότητες) και η γνωστική λειτουργία με τη χρήση της κλίμακας Montreal Cognitive Assessment (MoCA). Με το ερωτηματολόγιο αυτό θέλουμε να εκτιμήσουμε τη γενική νοητική κατάστασή σας σε ποικίλους τομείς, να αναγνωρίσουμε τη βαρύτητα της νοητικής δυσχέρειας, εφόσον υπάρχει, καθώς και να προσδιορίσουμε τους επιμέρους τομείς στους οποίους εκείνη μπορεί να εμφανίζεται πιο έντονα (οπτικοχωρική λειτουργία, ροή λέξεων εντός ενός λεπτού, αφαιρετική σκέψη, επανάληψη, προσοχή, κατονομασία, ικανότητα καθυστερημένης ανάκλησης λέξεων και προσανατολισμό σε χώρο και χρόνο).
3. Συλλογή δείγματος ούρων. Κατά τη διάρκεια κάθε επίσκεψης θα σας ζητηθεί να συλλέξετε μια ποσότητα ούρων περίπου 180-200 ml. Ηποσότητα αυτή των ούρων αντιστοιχεί φυσιολογικά σε 1-2 ουρήσεις, επομένως από την αρχή της επίσκεψής σας θα σας ζητηθεί να καταναλώσετε νερό και όταν θελήσετε να ουρήσετε θα σας δοθεί ειδικό δοχείο συλλογής.

Πλεονεκτήματα συμμετοχής στη Μελέτη

Η Μελέτη αυτή έχει ερευνητικούς σκοπούς. Εσείς προσωπικά δεν θα έχετε κάποιο

άμεσο θεραπευτικό όφελος από την συμμετοχή σας, και ούτε θα υπάρχει κάποιο άμεσο πλεονέκτημα σε σχέση με την διάγνωση ή θεραπεία της ασθένειάς σας. Υπάρχει η πιθανότητα έμμεσης ωφέλειας για εσάς, εφ' όσον η Μελέτη βοηθήσει στην ανακάλυψη καινούργιων θεραπειών ή βιολογικών δεικτών. Επιπλέον, με την συμμετοχή σας θα βοηθήσετε μελλοντικούς ασθενείς, που μπορεί να συμπεριλαμβάνουν και μέλη της οικογένειάς σας.

Πιθανά Προβλήματα συμμετοχής στην Μελέτη

Η Μελέτη αυτή είναι μη παρεμβατική και δεν αναμένεται να προκύψουν προβλήματα από τη συμμετοχή σας σε αυτή ούτε θα αλλάξει την αγωγή σας. Η μόνη ημι-επεμβατική πράξη είναι η αιμοληψία από την οποία μπορεί ορισμένοι ασθενείς να εμφανίσουν τοπικό πόνο, τοπική εκχύμωση, ζάλη ή λιποθυμία κατά τη διάρκεια ή μετά την αιμοληψία. Θρόμβοι ή λοιμώξεις σπάνια παρουσιάζονται.

Εμπιστευτικότητα και Ιδιωτικό απόρρητο

Η συμμετοχή σας στην Μελέτη καθώς και τα αποτελέσματα που θα προκύψουν είναι αυστηρά εμπιστευτικά. Βιολογικά δείγματα και κλινικά στοιχεία τα οποία θα συλλέξουμε, κωδικοποιούνται προκειμένου να βεβαιωθείτε ότι όλα τα δεδομένα είναι εμπιστευτικά. Η διαδικασία της κωδικοποίησης έχει ως εξής:

Μόλις γίνει η εισαγωγή σας στην Μελέτη, λαμβάνετε έναν ατομικό, μοναδικό κωδικό της Μελέτης. Όλα τα κλινικά στοιχεία και τα δείγματα που σας αναλογούν φέρουν αυτό τον κωδικό. Το αρχικό δείγμα (αίμα, ούρα), λοιπόν, κωδικοποιείται και δεν εμφανίζει κανένα στοιχείο της ταυτότητάς σας. Οι πληροφορίες που αφορούν τα δεδομένα των βιολογικών σας δεικτών αποθηκεύονται σε μια δεύτερη, ανεξάρτητη, ασφαλή, ηλεκτρονική βάση δεδομένων. Μόνον εξουσιοδοτημένο ερευνητικό προσωπικό έχει πρόσβαση στα κωδικοποιημένα δεδομένα των βιολογικών δεικτών σας. Το προσωπικό αυτό δεν έχει πρόσβαση στο όνομά σας ή τα κλινικά σας στοιχεία. Οι μόνοι που έχουν πρόσβαση είναι οι ιατροί της Μελέτης με τους οποίους συζητήσατε και από τους οποίους έγινε η κλινική αξιολόγηση.

Μετά την ολοκλήρωση της Μελέτης όλα τα δεδομένα αποθηκεύονται και αρχειοθετούνται σύμφωνα με τις ισχύουσες κατευθυντήριες γραμμές. Η μεταφορά και ανταλλαγή των στοιχείων σας ανάμεσα στους ερευνητικούς εταίρους της Μελέτης γίνεται πάντα με τη χρήση του ατομικού σας κωδικού. Στην περίπτωση της δημοσίευσης των αποτελεσμάτων της Μελέτης, το απόρρητο των προσωπικών σας δεδομένων είναι εγγυημένο.

Είναι πιθανό η χώρα στην οποία εδρεύει το εργαστήριο στο οποίο θα διαβιβαστεί το δείγμα του βιολογικού σας υλικού να μην έχει το ίδιο αυστηρά πρότυπα ασφάλειας προσωπικών δεδομένων. Η Ερευνητική Ομάδα όμως έχει μεριμνήσει ώστε όλα τα συνεργαζόμενα με αυτήν εξωτερικά εργαστήρια να τηρούν το ίδιο αυστηρές συνθήκες φύλαξης και προστασίας των προσωπικών σας δεδομένων.

Εάν θέλουμε να χρησιμοποιήσουμε τα βιολογικά σας δείγματα με οποιοδήποτε άλλον τρόπο από αυτόν που αναφέρεται σε αυτό το φύλλο πληροφοριών, θα πρέπει να ληφθεί πρώτα έγκριση της αντίστοιχης Επιτροπής Βιοηθικής.

Το παρόν πρωτόκολλο εφαρμόζει και συμμορφώνεται πλήρως με τις διατάξεις της κείμενης νομοθεσίας (Ενωσιακής και Ελληνικής) περί προστασίας προσωπικών δεδομένων. Αν δεν είστε ικανοποιημένος με τον τρόπο που διαχειρίζονται τα προσωπικά σας δεδομένα ή αν νομίζετε ότι γίνεται χρήση τους εκτός των αναφερομένων στην παρούσα ενημέρωση μπορείτε να υποβάλλετε παράπονο στη Αρχή Προστασίας Δεδομένων Προσωπικού Χαρακτήρα (www.dpa.gr).

Ασφάλιση

Η Μελέτη δεν προσφέρει κάποιο ειδικό είδος ασφάλισης. Παρακαλώ να ενημερώσετε αμέσως τον ιατρό της Μελέτης εάν νομίζετε ότι η συμμετοχή σας στην Μελέτη σας προξένησε κάποια σωματική βλάβη ή οδύνη. Ο ιατρός της Μελέτης θα κάνει ό,τι είναι δυνατόν για την ιατρική σας φροντίδα.

II. Συγκατάθεση του ασθενούς για τη συμμετοχή στη μελέτη:
«Βιοδείκτες βασισμένοι στην πρωτεΐνη LRRK2 στη νόσο Πάρκινσον»

- I. Παρακαλώ διαβάστε προσεκτικά αυτό το κείμενο.
- II. Αν υπάρχει κάτι που δεν καταλαβαίνετε όσον αφορά το πληροφοριακό σημείωμα ή θέλετε να ρωτήσετε οποιαδήποτε άλλη ερώτηση που σχετίζεται με τη Μελέτη, παρακαλώ επικοινωνήστε με τον ιατρό της Μελέτης, όπως αναφέρεται στο τέλος του πληροφοριακού σημειώματος
- III. Παρακαλώ ελέγξτε αν οι κάτωθι πληροφορίες είναι σωστές. Αν είναι έτσι και αν έχετε κατανοήσει τις εξηγήσεις που σας δόθηκαν και δεν έχετε άλλες απορίες τότε σημειώστε με ένα τικ στα κάτωθι κουτάκια και υπογράψτε.

Ενημέρωση

- Ο ιατρός της Μελέτης, που αναγράφεται στο παρόν έγγραφο, μου έχει δώσει γραπτό πληροφοριακό σημείωμα για τη Μελέτη με τον τίτλο που αναφέρεται πιο πάνω. Αυτό περιλαμβάνει τους πιθανούς κινδύνους, τα δικαιώματά μου σαν ασθενής και τι θα μου συμβεί στη Μελέτη. Μου δόθηκε η ευκαιρία να υποβάλω ερωτήσεις και να λάβω απαντήσεις για τις απορίες μου. Μου δόθηκε αρκετός χρόνος για να αποφασίσω για την συμμετοχή μου ή όχι στη Μελέτη. Γνωρίζω ότι ο ιατρός της Μελέτης θα είναι διαθέσιμος για επιπλέον ερωτήματα, εάν αυτά προκύψουν κατά την διάρκεια της Μελέτης.

Συμμετοχή

- Έχω καταλάβει ότι η συμμετοχή μου είναι εθελοντική και είμαι ελεύθερος να αποσυρθώ από τη Μελέτη οποιαδήποτε στιγμή το θελήσω, χωρίς να δώσω καμία εξήγηση και χωρίς αυτό να επηρεάσει την ιατρική μου φροντίδα ή τα νομικά δικαιώματά μου.

Ανωνυμία

- Έχω αντιληφθεί ότι η αναγνώριση των βιολογικών δειγμάτων μου και των πληροφοριών που με αφορούν θα γίνεται με έναν κωδικό που θα δοθεί σε αυτά και ότι έτσι εξασφαλίζεται η ανώνυμη συμμετοχή μου στην Μελέτη.

Ιατρικός φάκελος/ιατρικά δεδομένα

- Έχω καταλάβει ότι ο ιατρικός μου φάκελος και τα στοιχεία από το ιατρικό ιστορικό μου μπορεί να αναζητηθούν και να μελετηθούν από εξουσιοδοτημένα άτομα της ερευνητικής ομάδας που έχει σχεδιάσει και θα εκτελέσει τη Μελέτη. Αυτό θα γίνει με εμπιστευτικότητα. Δίδω την άδεια μου σε αυτά τα άτομα για την πρόσβασή τους στον ιατρικό μου φάκελο. Τα στοιχεία του φακέλου μου θα εμπλουτιστούν κατά την διάρκεια της συμμετοχής μου στην Μελέτη με επιπλέον στοιχεία, που θα αφορούν την κλινική μου κατάσταση. Στον ιατρικό μου φάκελο δεν θα αναφέρονται στοιχεία από την μελέτη των βιολογικών δεικτών που θα προκύψουν κατά την

διάρκεια της Μελέτης. Στοιχεία από τον ιατρικό μου φάκελο θα χρησιμοποιηθούν σε επιπλέον αρχεία (ηλεκτρονικά ή γραπτά), όπου δεν θα αναφέρεται το όνομά μου, παρά μόνον ο κωδικός που θα αντιστοιχεί στα δεδομένα μου. Τα δεδομένα σε αυτά τα επιπλέον αρχεία θα αναλυθούν στα πλαίσια της Μελέτης με στατιστικές μεθόδους. Τα αρχεία αυτά θα αρχειοθετηθούν και θα είναι διαθέσιμα σε ερευνητές και μετά το πέρας της μελέτης, για τουλάχιστον 20 έτη. Η αντιστοίχιση του ιατρικού μου φακέλου με τον κωδικό θα υπάρχει μόνο σε ειδικό έγγραφο που θα είναι σε ξεχωριστό αρχείο, που θα φυλάσσεται από τον υπεύθυνο της Μελέτης και δεν θα είναι διαθέσιμο σε άλλα άτομα.

Αποθήκευση βιολογικών υλικών

- Δέχομαι τα βιολογικά μου δείγματα να αποθηκευτούν από τον Ιατρό της Μελέτης και να σταλούν σε συνεργαζόμενα κέντρα για περαιτέρω ανάλυση. Δέχομαι τα δείγματα που δεν θα χρησιμοποιηθούν να παραμείνουν αποθηκευμένα για 20 έτη. Σε οποιαδήποτε στιγμή, μπορώ να ζητήσω να καταστραφούν τα βιολογικά μου δείγματα.

Διαγνωστικό/θεραπευτικό όφελος

- Έχω αντιληφθεί ότι αυτή την στιγμή οι εξετάσεις που θα διενεργηθούν δεν σχετίζονται με κάποιο διαγνωστικό ή θεραπευτικό όφελος, και, για αυτό τον λόγο, δεν θα ενημερωθώ για αποτελέσματα που θα προκύψουν στα πλαίσια της Μελέτης σχετικά με τα προσωπικά κλινικά μου χαρακτηριστικά ή τους βιολογικούς μου δείκτες (εκτός από αποτελέσματα γενετικής ανάλυσης, στην περίπτωση που συμφώνησα να διενεργηθεί και δέχτηκα να ενημερωθώ για τα αποτελέσματά της). Σε περίπτωση που στο παρελθόν δεν δέχτηκα να ενημερωθώ για τα αποτελέσματα των γενετικών εξετάσεων, αλλά ανέπτυξα συμπτώματα νόσου Πάρκινσον κατά την διάρκεια της συμμετοχής μου στην παρούσα Μελέτη, θα έχω την δυνατότητα να ενημερωθώ για τα αποτελέσματα αυτά στα πλαίσια της παρούσας Μελέτης.

□

Αντίγραφα εγγράφων Μελέτης

- Έχω παραλάβει ένα αντίγραφο του πληροφοριακού σημειώματος και της παρούσας συγκατάθεσης.

Συναίνεση

- Ως εκ τούτου δέχομαι να συμμετάσχω στη παρούσα Μελέτη. Συγκεκριμένα, δέχομαι να συμμετάσχω στις κλινικές, γενετικές και βιοχημικές εξετάσεις που αποτελούν μέρος της παρούσας Μελέτης, και συνυπογράφω κατωτέρω. Παρακάτω αναφέρονται επιμέρους εξετάσεις στις οποίες δέχομαι να συμμετάσχω.

☐ Ναι ☐ Όχι	Δέχομαι να διενεργηθεί φλεβοτομή για την λήψη αίματος. Το αίμα θα χρησιμοποιηθεί για την απομόνωση λευκών αιμοσφαιρίων και πλάσματος, που θα αναλυθούν στο πλαίσιο της Μελέτης.
☐ Ναι ☐ Όχι	Δέχομαι να συλλέξω ούρα και να τα παραδώσω στον ιατρό της Μελέτης. Τα ούρα θα αναλυθούν στο πλαίσιο της Μελέτης.
☐ Ναι ☐ Όχι	Δέχομαι να χρησιμοποιηθούν οι πληροφορίες σχετικά με τα δημογραφικά και κλινικά μου δεδομένα και τα βιολογικά μου δείγματα

Ονοματεπώνυμο συμμετέχοντα Ημερομηνία Υπογραφή

Έχω ενημερώσει τον/την ασθενή/φορέα/άτομο ελέγχου για την συμμετοχή του/της στην Μελέτη και έχω παραδώσει το Πληροφοριακό Σημείωμα.

Ονοματεπώνυμο ιατρού Μελέτης Ημερομηνία Υπογραφή

Σε περίπτωση που ο/η συμμετέχων δεν έχει την ικανότητα να αποφασίσει για την συμμετοχή του/της στην μελέτη, την απόφαση λαμβάνει νόμιμος εκπρόσωπός του/της, μετά από την ίδια ως άνω διαδικασία, και συνυπογράφει.

Ονοματεπώνυμο νόμιμου Ημερομηνία Υπογραφή
εκπροσώπου συμμετέχοντα

III. Scientific Publications

ARTICLE OPEN



Distinct profiles of LRRK2 activation and Rab GTPase phosphorylation in clinical samples from different PD cohorts

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Despite several advances in the field, pharmacodynamic outcome measures reflective of LRRK2 kinase activity in clinical biofluids remain urgently needed. A variety of targets and approaches have been utilized including assessments of LRRK2 itself (levels, phosphorylation), or its substrates (e.g. Rab10 or other Rab GTPases). We have previously shown that intrinsic kinase activity of LRRK2 isolated from PBMCs of G2019S carriers is elevated, irrespective of disease status. In the present study we find that phosphorylation of Rab10 is also elevated in G2019S carriers, but only those with PD. Additionally, phosphorylation of this substrate is also elevated in two separate idiopathic PD cohorts, but not in carriers of the A53T mutation in α -synuclein. In contrast, Rab29 phosphorylation was specifically reduced in urinary exosomes from A53T and idiopathic PD patients. Taken together, our findings highlight the need for the assessment of multiple complimentary targets for a more comprehensive picture of the disease.

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INTRODUCTION

The kinase activity of leucine-rich repeat kinase 2 (LRRK2) has emerged as the primary target for both therapeutic development and biomarker design in Parkinson's disease (PD). Both cellular and animal models have demonstrated that neurodegeneration induced by mutant forms of LRRK2 is kinase activity-dependent (e.g. ref. ¹). Similarly, early biomarker studies, assessing LRRK2 auto-phosphorylation (pS1292-LRRK2) in urinary exosomes, have indicated that LRRK2 activity may be elevated during the clinical progression of LRRK2-associated, as well as idiopathic, PD (iPD)^{2,3}. In addition to auto-phosphorylation of LRRK2 and its intrinsic kinase activity, which are measures of distinct aspects of its kinase function, other readouts of the activation state of LRRK2 include phosphorylation of endogenous substrates, such as members of the Rab family of small GTPases⁴. Extending these findings, the group of Greenamyre and colleagues⁵ applied a novel proximity ligation assay to detect pS1292-LRRK2 and pT73-Rab10 in ventral midbrain of post mortem tissue from iPD patients. In subsequent studies, we have reported that the in vitro kinase activity of purified LRRK2 from peripheral blood mononuclear cells (PBMCs) of G1029S-LRRK2 mutation carriers is elevated⁶. Alternatively, while phosphorylation at the S935 site within the N-terminal region of LRRK2 is a robust measure of target engagement (e.g. refs. ^{7,8}), it does not completely track with kinase activity of LRRK2⁹.

Rab10 phosphorylation has been assessed in various PD cohorts, however the findings have been mixed. In a small proof-of-concept study, Rab10 phosphorylation (T73) in neutrophils was not significantly changed in carriers of the G2019S LRRK2 mutation¹⁰; and in PBMCs from iPD patients, pT73-Rab10 was not changed compared to healthy controls, despite being correlated with levels of certain cytokines¹¹. Interestingly, in neutrophils from carriers of the less common R1441G mutation in LRRK2, a significant increase in pT73-Rab10 is detected in comparison to healthy controls, irrespective of disease status¹².

This is consistent with findings reported in transgenic mice expressing R1441C or G2019S, where a robust increase in Rab10 phosphorylation was observed in R1441C but not G2019S mice¹³. Most recently, using a proprietary antibody raised against pT73-Rab10, Wang and colleagues reported that phosphorylated Rab10 levels, normalized to total LRRK2, are increased in PBMCs of affected carriers of the G2019S mutation⁸ and are reduced in cells treated ex vivo with LRRK2 kinase inhibitors.

In the present study, we find increased Rab10 phosphorylation in iPD patients and affected G2019S-LRRK2 carriers only, whereas levels in healthy carriers and A53T-SNCA PD patients are not changed from healthy controls. Additionally, phosphorylation of Rab29 is decreased in urinary exosomes from SNCA mutation carriers and iPD patients. Thus, it appears that alterations in peripheral LRRK2-dependent Rab phosphorylation are not uniformly linked to either disease status or genotype.

RESULTS

Phosphorylation of Rab10 is increased in PBMCs of iPD and G2019S-LRRK2 carriers with PD

By Western immunoblotting, we assessed levels of pT73-Rab10 and pT71-Rab29 in PBMC extracts from each group. In Fig. 1a, a representative immunoblot showing levels of phosphorylated (T73) and total Rab10 from each group from the Columbia cohort samples is shown, along with quantification of the bands. In Fig. 1b, we show similar representative blots from the Athens cohort and their accompanying quantification. When normalized to total Rab10 levels, we found a significant increase in pT73-Rab10 in idiopathic PD (iPD) from both the Columbia cohort, as well as the Athens cohort, compared to healthy control subjects (Fig. 1a, b). We treated a parallel set of PBMCs with the LRRK2 inhibitor MLI2, and found a significant reduction in pRab10 levels compared to untreated cells (Supplementary Fig. 2a), indicating that in these cells Rab10 phosphorylation is largely mediated by LRRK2 kinase

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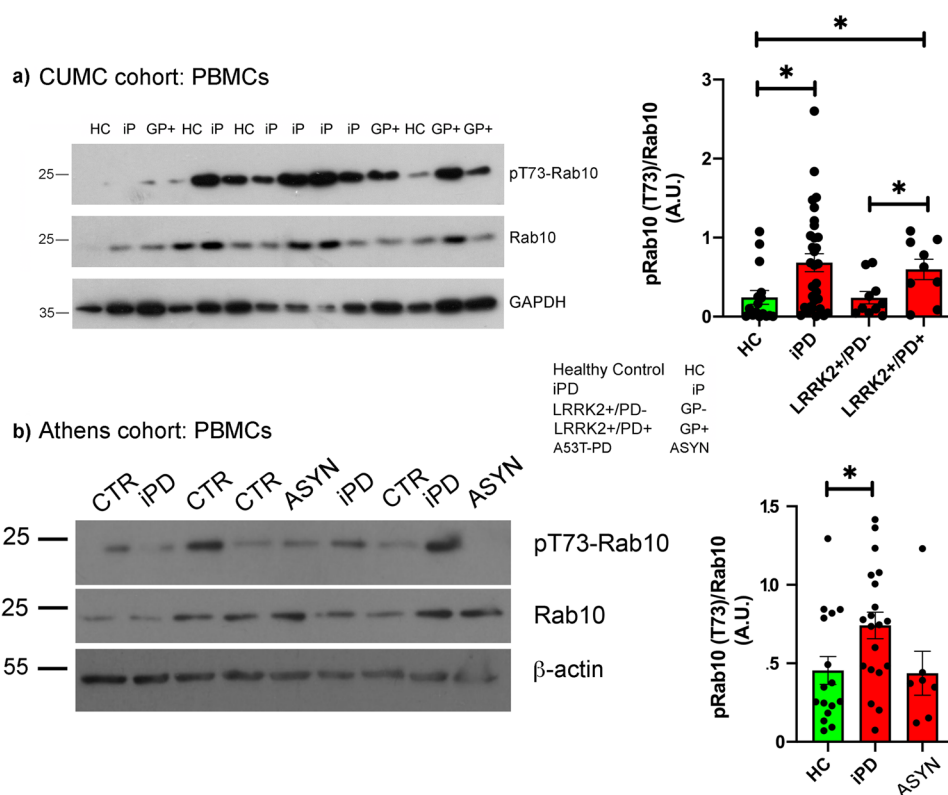


Fig. 1 Phosphorylation of Rab10 is elevated in PBMCs from idiopathic PD and affected carriers of the G2019S mutation in LRRK2. Extracts of PBMCs from the indicated subject groups were separated by SDS-PAGE and probed for phosphorylated (T73) and total Rab10, as well as GAPDH. **a** Representative western immunoblots showing pT73-Rab10 and total Rab10 from PBMCs of the CUMC cohort; with the quantification of the band intensity shown to the right. ANOVA was performed to identify significant differences between groups, with Tukey's post-foc tests. $*p < 0.05$. **b** PBMC extracts were separated by SDS-PAGE. A representative western immunoblot probed for pT73-Rab10 and total Rab10 from PBMCs of the Athens cohort; with the band intensities quantified and presented in the panel to the right. For normalization, the band intensity of pT73-Rab10 was then normalized to total Rab10. Error bars in all data plots represent SEM. ANOVA was performed to identify significant differences between groups, with Tukey's post-foc tests. $*p < 0.05$.

activity. Like LRRK2 expression⁶, expression of Rab10 (total) was not significantly altered in either iPD cohort. As an alternative approach, similar to what has been reported by Wang and colleagues⁸, we normalized pRab10 to total LRRK2 expression; however, while the same trends were retained (i.e. G2019S+ PD+ and iPD levels were slightly increased) the differences were not statistically significant (Supplementary Fig. 1C).

In the two G2019S-*LRRK2* carrier groups, we found a divergence in Rab10 phosphorylation that was strictly linked to disease status. Affected carriers of this mutation exhibited a significant increase in normalized pT73-Rab10 levels, similar to iPD patients, compared to healthy controls as well as G2019S+ PD−; whereas pRab10 levels were not different from control subjects in non-manifesting G2019S-*LRRK2* carriers (Fig. 1a, b). We have previously shown from the same cohort⁶, that the in vitro kinase activity of *LRRK2* purified from PBMCs of both G2019S carrier groups is significantly increased compared to healthy control and iPD patients. Thus, in a cellular milieu, the disease context is clearly impacting the phosphorylation of *LRRK2* substrates, even in the presence of a hyperactive enzyme. In these cohorts however, we did not observe significant correlations between phosphorylated Rab10 levels and multiple clinical outcomes (i.e. UPDRS-III, MoCA, disease duration). Finally, in PBMCs from affected carriers of the A53T mutation in α -synuclein (ASYN), phosphorylation of Rab10 was unchanged in PBMCs compared to healthy controls (Fig. 2a, b).

In addition to Rab10 phosphorylation, we assessed the induction of phosphorylation of another LRRK2 substrate, Rab29, in PBMCs from each group. We could readily detect robust expression of Rab29 in PBMCs from each group, and this did not

significantly differ between each group. Interestingly, in most samples, we could not detect phosphorylated Rab29 (T71), even in the presence of a signal boost reagent (SignalBoost Immunoreaction, Sigma; not shown), suggesting that within PBMcs, Rab29 is not a particularly prominent LRRK2 substrate, either in the healthy control population or in any of the PD cohorts; or that specific Rab29 phosphatases are particularly active in these cells.

Changes in Rab10 and Rab29 phosphorylation in urinary extracellular vesicles/exosomes

In extracts of urinary extracellular vesicles (EVs), the detection of LRRK2 was highly variable; in many samples, total and pS1292-LRRK2 was un-detectable (Supplementary Fig. 2b, c), even in the presence of the SignalBoost detection reagent. However, we were able to easily detect both total and pT73-Rab10 in extracts of EVs by Western immunoblotting (Fig. 2a). The pattern of Rab10 phosphorylation differed in urinary EVs; where, in contrast to PBMcs, phosphorylated Rab10, normalized to total Rab10, in EVs from iPD and A53T-ASYN patients were unchanged compared to healthy controls (Fig. 2b). Interestingly, whereas we could not detect phosphorylated Rab29 in extracts of PBMcs from this cohort, we were able to detect pT71-Rab29 in urinary EVs; and found a significant decrease in pRab29 (normalized to total Rab29) in EVs from A53T-PD patients compared to healthy controls (Fig. 2c). Phosphorylated Rab29 levels in urinary EVs from iPD patients were also reduced compared to healthy controls (Fig. 2c); however the drop in pRab29 levels did not correlate with any of the clinical outcomes reported (e.g. UPDRS-III, MoCA, or disease duration).

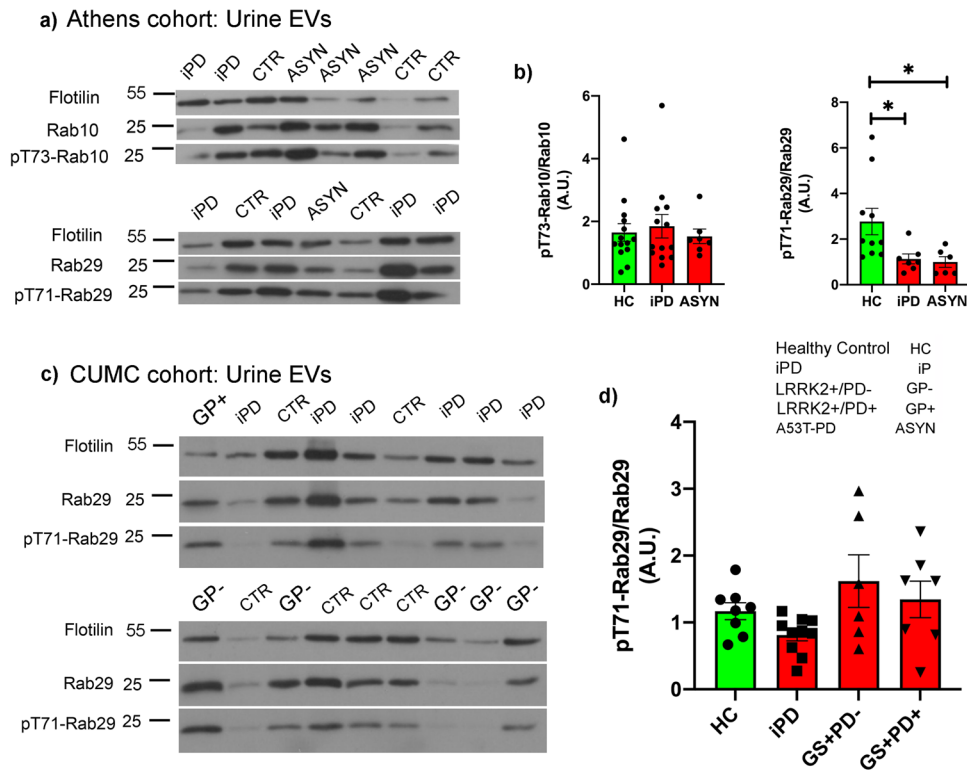


Fig. 2 Phosphorylation of Rab10 is unaffected in urinary EVs but phosphorylation of Rab29 is decreased in two PD cohorts. EVs from urine were purified by ultracentrifugation, and extracts separated by SDS-PAGE. The membranes were probed with total or phosphorylated Rab10 and 29, and flotillin as a marker for EVs. Band intensities of phosphorylated Rab10 or Rab29 were normalized to total Rab. **a, c** Representative blots showing phosphorylated Rab10 and Rab29; and quantified in **(c, d)**. Error bars in all data plots represent SEM. ANOVA was performed to identify significant differences between groups, with Tukey's post-hoc tests. * $p < 0.05$.

We did observe a slight decrease in pRab29 in urine EVs of iPD patients from the CUMC cohort, similar to the Athens cohort, which only attained statistical significance following a two-tailed *t*-test (not shown), and not following the ANOVA performed on the full data set. Interestingly, we did not observe a correlation between levels of pRab10 in PBMCs and urine EVs, in either cohort. The reason for this is likely due to the different cellular sources of the Rab10 in each sample. There are multiple cell types in the PBMC population that express detectable levels of Rab10¹⁰, and similarly it is not possible, in the preparations we employed, to determine the cellular source of the EVs recovered from the patient urine samples. A more targeted study of specific blood cell types and immuno-isolated EVs (e.g. from urine, plasma, or even CSF) would be better suited to reveal such a correlation.

In addition to PBMCs and urine, some study participants from the Athens cohort were asked to provide an additional sample from which we purified CD14+ monocytes. A separate manuscript describing in more detail these samples, focusing on changes in LRRK2 levels and activity, is being prepared; but in the current study we also assessed changes in Rab10 phosphorylation. In neither the iPD study group, nor the A53T-ASYN PD group, were we able to detect significant changes in phosphorylation of Rab10 compared to healthy controls. These cells express high levels of LRRK2¹⁰; however its phosphorylation of Rab10 at Thr73 appears unchanged in either PD study group, at least among the small sample size assessed here (Supplementary Fig. 3). We were not able to assess Rab10 phosphorylation in carriers of the G2019S mutation in the LRRK2 gene; where we might expect to detect elevated phosphorylation within the affected group, as we have in PBMCs from this cohort.

DISCUSSION

In the present study, we assessed Rab GTPase phosphorylation in PBMCs and urinary exosomes from multiple PD cohorts collected at different clinical centers. We recently reported several LRRK2-based outcome measures in a cohort of G2019S-LRRK2 carriers (both healthy and affected) from the CUMC-Movement Disorders center. In PBMCs from these individuals, we found a significant elevation in phosphorylation of the LRRKtide peptide substrate in both G2019S carrier groups, compared to healthy controls and idiopathic PD patients⁶. This is not surprising given the conserved motif that this mutation lies within in the LRRK2 kinase domain (e.g. ref. ¹⁴). In the same report, we could not detect changes in the *in vitro* kinase activity of LRRK2 isolated from iPD PBMCs⁶.

In PBMCs from G2019S carriers, we report here increased phosphorylation of Rab10 (T73) *only* in affected carriers of this mutation, healthy G2019S carriers exhibited pT73-Rab10 levels similar to healthy controls. Thus, the isolated enzyme bearing the Gly to Ser substitution in the critical DYG kinase domain motif exhibits elevated intrinsic kinase activity, regardless of disease status⁶; however, in a cellular context the disease status clearly interacts with the genotype to modulate Rab10 phosphorylation, at least in peripheral immune cells. In other words, *in vitro* kinase activity is a good predictor of LRRK2 mutation status, but not of disease status. In another recent study, combining western immunoblotting and targeted mass-spectrometry measures of phosphorylated Rab10, it was reported that T73-Rab10 levels in a small sample of carriers of the R1441G were significantly elevated in neutrophils compared to healthy control subjects¹². Unlike the present study, phosphorylated Rab10 levels from carriers of the G2019S-LRRK2 mutation were not different from healthy controls, regardless of disease status¹²; however in an earlier study also

Table 1. Summary of demographics of study participants (Athens cohort).

	Healthy controls (HC)	Idiopathic PD (iPD)	A53T-SNCA PD	<i>p</i> value	Statistical tests
Gender (M:F)	7:9	14:6	4:4		
Age	55 (36–75)	72 (47–85)	51 (30–73)		
Age at onset	na	66 (42–80)	46 (27–68)	<i>p</i> = 0.0011	MWU (iPD vs A53T-PD)
LEDD	na	629 (0–1865)	758 (150–1500)	ns	MWU (iPD vs A53T-PD)
UPDRS-III	0	30 (0–91)	31 (9–53)	ns	MWU (iPD vs A53T-PD)
MoCA	26 (17–30)	19 (4–26)	26 (16–30)	HC vs. iPD (<i>p</i> = 0.0025) HC vs. A53T (ns)	MWU
H&Y	0	2 (0–4)	2 (1–3)	ns	MWU (iPD vs A53T-PD)

H&Y Hoehn & Yahr PD scale, MoCA Montreal Cognitive Assessment, LEDD levodopa equivalent daily dosage, UPDRS-III united PD rating scale (part 3).

using an MS-based approach, pT73-Rab10 levels in neutrophils were approximately 2-fold higher in a set of G2019S carriers¹⁵. We did not detect changes in Rab10 phosphorylation in CD14+ monocytes in either the iPD or the A53T-ASYN patient groups, suggesting that the cellular source of increased pRab10 in the iPD and G2019S+PD+ PBMCs is not this subset of monocytes, but perhaps neutrophils (e.g.¹²), or other monocyte subsets, such as CD16+ monocytes which have been shown to exhibit increased LRRK2 expression in PD¹⁶. Additionally, using a novel phospho-specific Rab10 antibody in an ELISA-based outcome measure, Wang and colleagues reported slightly elevated pRab10, normalized to LRRK2 levels, in PBMCs of both healthy and affected G2019S carriers⁸. It should be noted that in this study, the authors reported a slight but significant decrease in LRRK2 expression in both G2019S-LRRK2 groups, which may account for the elevated pRab10 levels when normalized to LRRK2 instead of total Rab10. Nevertheless, while the current study remains semi-quantitative in its approach, the study by Wang and colleagues represents an important advance in the field in terms of more quantitative assessments of changes in LRRK2 pathway signaling in PD.

In PBMCs from iPD or A53T-PD patients, we do not detect changes in the intrinsic kinase activity of isolated LRRK2 (ref. ⁶; and manuscript in preparation); whereas in two independent cohorts (CUMC and Athens), we find increased phosphorylation of Rab10 in PBMCs from iPD patients. Not surprisingly, there is considerable variation in phosphorylated Rab10 levels in these clinical samples, which is reflective of course of LRRK2 kinase activity but also potentially other kinases, as well as the activity of PPM1H, a phosphatase implicated in Rab10 de-phosphorylation¹⁷. Further, deeper analyses should be performed to determine if specific subsets of patients exhibit increased Rab10 phosphorylation, which would likely be correlated with other biochemical outcomes or other specific clinical measures. In this study, however, we did not observe any correlations with clinical outcomes (e.g. UPDRS-III and others). Previous reports have not revealed differences in Rab10 phosphorylation in this patient group (e.g. ref. ¹¹); the reasons for this discrepancy are unclear, however may be related to the normalization methods taken in each study. Rab10 phosphorylation is reported to be increased in post-mortem brain from iPD patients⁵; thus, taken together with our present findings, it suggests that increased phosphorylation of this key substrate, and by extension LRRK2 kinase activity, is a systemic phenomenon in this disease cohort. The lack of significant increases in Rab10 phosphorylation in A53T-PD patients suggests that there are multiple PD progression pathways, depending on the form of the disease. Alternatively, in this specific patient group, with an earlier age of disease onset (see Table 1), it is possible that a transient increase in Rab10 phosphorylation occurred at an earlier point in the disease course. And finally, given that the sample number was significantly less in this patient group, it is also possible that with additional samples subtle changes in Rab10 phosphorylation in

PBMCs would be revealed. It is not known at this point if there are detectable changes in Rab10 phosphorylation in brains of A53T-PD patients; however we are currently assessing neuronally derived exosomes for changes in LRRK2 and Rab phosphorylation. Additionally, a longitudinal study with sufficiently large patient groups assessing multiple markers is critically needed to address these questions.

A second key finding we report here is the loss of phosphorylation of Rab29 (Thr71) in urinary exosomes from iPD and A53T-PD patients. Recently, it was reported by two independent groups that over-expression of Rab29 can trigger the recruitment of LRRK2 to intracellular membranes and the trans-Golgi network (TGN) leading to its activation^{18,19}; suggesting a regulatory cycle between LRRK2 activity and Rab29. In our clinical biofluid samples, the loss of phosphorylation of Rab29 in urine exosomes from iPD and A53T-PD patients, releasing its inhibition, would be consistent with increased activation of LRRK2 (e.g. increased pT73-Rab10 in the iPD group). While we could not detect changes in phosphorylation of Rab10 in this cohort, the reduction in Rab29 phosphorylation is the first indication of possible changes in LRRK2 activation in clinical samples from PD patients carrying the A53T-SNCA mutation; and bolsters the notion that LRRK2 function plays an important role in PD in general. A summary of our findings in the current study, in the context of our previously reported findings from one of the two cohorts (from CUMC) is presented in Table 2. Our findings reinforce the need to expand our pallet of validated LRRK2-pathway outcome measures to include additional targets as well as multiple biofluid types²⁰.

METHODS

Study participants

The samples that we assessed were collected as part of two separate biomarker studies. The demographics of a subset of the clinical cohort (healthy controls, iPD, G2019S-LRRK2/PD+ & G2019S-LRRK2/PD-) followed at Columbia University Medical Center (New York, USA) have been reported in detail in a separate manuscript⁶, and are summarized in Table 1. The second cohort (healthy controls, iPD, & A53T-SNCA PD) was derived from patients followed at the Departments of Neurology at Henry Dunant Hospital Center (Athens, Greece) and Aiginiteio University Hospital (Athens, Greece). All samples were collected, processed and coded at the time of collection so that the analyses of the samples were performed by researchers blinded to the PD and mutation status. The study was approved by the institutional ethical review boards of both Columbia University (RNA) as well as Biomedical Research Foundation of the Academy of Athens (BRFAA; HJR), and all participants signed written informed consent. Thus, in this study we have included two independent groups of healthy controls and iPD patients. Demographics and key clinical features of the Athens cohort are summarized in Table 3. The samples from each cohort were analyzed independently. In some cases, fewer samples were included in a specific analysis than were originally collected due to technical issues with detection of the specific target in the sample matrix.

Table 2. Summary of demographics of study participants (CUMC cohort).

	Healthy controls (HC)	Idiopathic PD (iPD)	LRRK2+/PD−	LRRK2+/PD+	<i>p</i> value	Comparisons
Sex (M:F)	8:12	18:12	4:5	4:5	ns	
Age	68.3 (57–85)	67.5 (45–86)	57.1 (38–69)	74 (64–90)	<i>p</i> < 0.01–0.05	GS+/PD− vs. all groups
Age at onset	na	60.5 (27–84)	na	64.6 (54–83)	ns	
LEDD	na	466.3 (0–1520)	na	631.3 (300–1625)	ns	
UPDRS-III	0	18.9 (5–46)	0	20.6 (12–28)	ns	
MoCA	27.8 (24–30)	26.4 (15–30)	28.4 (27–30)	26.5 (22–29)	<i>p</i> < 0.05	iPD vs. GS+/PD−

H&Y Hoehn & Yahr PD scale, MoCA Montreal Cognitive Assessment, LEDD levodopa equivalent daily dosage, UPDRS-III united PD rating scale (part 3).

Table 3. Summary of LRRK2-specific changes in two PD cohorts.

	Total LRRK2	In vitro kinase activity	pS935-LRRK2	pS1292-LRRK2 (WB)	pT73-Rab10 (WB)	pT71-Rab29 (WB)
Healthy Control (HC)	Baseline	Baseline	Baseline	Detectable only	Baseline	Baseline
Idiopathic PD (iPD)	Like HC	Like HC	Elevated (2 cohorts; Melachroinou et al., 2020; manuscript in prep.)	Detectable only	Elevated (2 cohorts)	Decreased (Ur-EVs)
G2019S+ PD+	Like HC	Elevated (Melachroinou et al., 2020)	Slight decrease (Melachroinou et al., 2020)	Detectable only	Elevated	ND
G2019S+ PD−	Like HC	Elevated (Melachroinou et al., 2020)	Slight decrease (Melachroinou et al., 2020)	Detectable only	Like HC	ND
A53T-SNCA PD	Manuscript in prep.	Manuscript in prep.	Manuscript in prep.	Detectable only	Like HC	Decreased (Ur-EVs)

For example, in the samples where signal from the total protein (for normalization) was missing, these were excluded from the analyses.

PBMC & urinary exosome isolation

PBMCs were isolated from whole blood collected in Heparin-coated Vacutainer collection tubes (BD), using protocols established in our recent work⁶. Some aliquots were divided into two samples and treated with DMSO or the LRRK2 inhibitor ML-2, then collected/washed in PBS and snap frozen as a cell pellet in dry ice, and stored at −80 °C. Urine (~50–200 ml) was collected from participants in the morning of their clinic visit and tested for urinary infections.

Exosomes were isolated as described from the group of West and colleagues³, and lysed under the same conditions as PBMCs. Briefly, an initial centrifugation took place for 30 min, at 4500 g, at 4 °C for debris clearance and the supernatant was stored at −80 °C in 50 ml falcon tubes. After quickly thawing the urine in a 42 °C waterbath, the EVs were isolated by ultracentrifugation (Sorval Discovery, 100 SE) at 100,000 g, for 1 h at 4 °C. EV pellets were lysed with 50 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 270 mM Sucrose, 1% Triton + 1X protease/phosphatase inhibitors added fresh), for 1 h on ice. Lysates were then centrifuged at 13,000 rpm, for 15 min at 4 °C and the supernatant was collected and stored at −80 °C. Parallel EV pellets from sample preps were resuspended in PBS after ultracentrifugation for TEM imaging (shown in Supplementary Fig. 2d). For the preparation of urinary exosomes for visualization by transmission electron microscopy (TEM), the exosome pellets were resuspended in PBS, and 2 µl of exosome suspension removed for imaging. Apart from the TEM confirmation of EV size and shape, and positive immunoreactivity for the exosomal marker flotillin, we confirmed that extracts of urine EVs were also positive for the exosomal markers TSG101 (Supplementary Fig. 2e).

Where possible, in the Athens cohort, we collected CD14+ monocytes in addition to PBMCs and urine. Blood was collected in K₂-EDTA Vacutainer collection tubes (BD) and 15 ml was transferred to a 50 ml falcon and 750 µl "Isolation Cocktail" from the EasySep™ Direct Human Monocyte Isolation Kit (StemCell Technologies) was added. "RapidSphere Magnetic Beads" from kit were vortexed for 30 s before use and added to the blood sample. Blood was mixed gently by tube inversion and incubated for 5 min, at RT. The volume was increased to 50 ml with 1 mM EDTA-PBS Ca²⁺/Mg²⁺ free (Gibco), and the solution was gently mixed. The tube was placed into a magnet (StemCell Technologies) without the lid, for 10 min,

to immobilize the magnetic beads along the wall of the centrifuge tube. The enriched monocyte cell suspension was carefully removed (leaving ~10 ml behind) into a new 50 ml falcon, where 750 µl of fresh magnetic beads were added and tube was gently mixed followed by 5 min RT incubation. The cell suspension was again removed (leaving ~5 ml behind) and to ensure complete removal of magnetic beads, the tube was placed in the magnet for another 10 min, at RT. Purified CD14+ monocytes were then transferred to a new falcon (leaving ~5 ml behind) and the 30 ml suspension was increased with 1 mM EDTA-PBS Ca²⁺/Mg²⁺ to a final volume of 40 ml. Centrifugation for 5 min, at 335 g, at RT followed and the cell pellet was washed once with 1X PBS and stored at −80 °C. Prior to biochemical analysis, pellets were lysed with 50 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 270 mM Sucrose, 1% Triton + 1X protease EDTA-free/phosphatase cocktail inhibitors (Roche) added fresh), for 30 min on ice. Lysates were then centrifuged at 45,000 rpm, for 1 h, at 4 °C and S/N was collected and stored at −80 °C.

For western immunoblotting, protein samples were separated by SDS-PAGE, and the membranes blocked in either 5% Milk/TBST (for Rab10, Rab29, GAPDH, LRRK2, and Flotilin-1) or in 5% BSA/TBST (for pS1292-LRRK2, pT73-Rab10 and pT71-Rab29), for 1 h at RT. The primary antibodies used were as follows: Rab10 (ab237703 Abcam, rabbit, 1:1000), Rab29 (ab199644 Abcam, rabbit, 1:1000), Flotilin-1 (sc-133153 Santa Cruz, mouse, 1:1000), LRRK2 (ab133474 Abcam rabbit, clone c41-2, 1:1000), pT73-Rab10 (ab230261 Abcam, rabbit, 1:1000) and pT71 Rab29 (ab241062 Abcam, rabbit, 1:1000), and pS1292-LRRK2 (ab203181 Abcam, clone MJF-19-7-8, 1:1000), shaking overnight at 4 °C. Initially, full-length membranes were probed with a primary antibody (as indicated, representative blot is shown in Supplementary Fig. 1a), and then stripped before re-probing. In subsequent immunoblots, the membranes were cut horizontally around the molecular weight of the specific protein, to preserve the limited volume of the clinical samples, and probe each membrane with multiple antibodies. In some cases, where the membranes were probed with anti pS1292-LRRK2 antibodies, we included Signal Boost Immunoreaction Reagent (Sigma) in the antibody diluent for both the primary and secondary antibody incubation steps. Membranes were then washed 3x for 10 min in 1X TBST and incubated with HRP-conjugated secondary antibodies (Millipore AP132P anti-rabbit or Millipore AP124P anti-mouse, 1:5000), for 1 h at RT. Three more washes followed and membranes were exposed to ECL and developed using an automatic film developer. Films

were scanned and quantified using ImageJ, with the final Figures compiled using Adobe Photoshop.

For quantification and normalization of the phosphorylated protein, the ratio of band intensity of phosphorylated:total protein was determined. Because of the semi-quantitative nature of western immunoblotting, band intensities were measured from films taken from similar exposures wherever possible. The samples were run in a blinded fashion (i.e. coded), and band intensities were obtained by an independent researcher, before the sample ID's were decoded. Band intensities of phosphorylated Rab10 were first normalized to total Rab10 expression; and, alternatively to total levels of LRRK2 determined by ELISA⁶.

Statistical analyses

For statistical comparison of the different study groups, we employed ANOVA with Tukey's post-hoc multiple comparisons, or if required non-parametric Kruskal–Wallis tests & Dunn's post-hoc test; or two-tailed t-tests where indicated.

DATA AVAILABILITY

Non-identifying data generated during this study can be made available from the corresponding author upon reasonable request.

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REFERENCES

- Greggio, E. et al. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol. Dis.* **23**, 329–341 (2006).
- Fraser, K. B., Moehle, M. S., Alcalay, R. N. & West, A. B., Consortium, L. C. Urinary LRRK2 phosphorylation predicts parkinsonian phenotypes in G2019S LRRK2 carriers. *Neurology* **86**, 994–999 (2016).
- Fraser, K. B. et al. Ser(P)-1292 LRRK2 in urinary exosomes is elevated in idiopathic Parkinson's disease. *Mov. Disord.* <https://doi.org/10.1002/mds.26686> (2016).
- Steger, M. et al. Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a subset of Rab GTPases. *Elife* **5**, <https://doi.org/10.7554/eLife.12813> (2016).
- Di Maio, R. et al. LRRK2 activation in idiopathic Parkinson's disease. *Sci Transl Med.* **10**, <https://doi.org/10.1126/scitranslmed.aar5429> (2018).
- Melachroinou, K. et al. Elevated in vitro kinase activity in PBMCs of LRRK2 G2019S carriers: a novel ELISA-based method. *Mov. Disord.* **35**, 2095–2100 (2020).
- Dzambo, N. et al. Inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser(910)/Ser(935), disruption of 14-3-3 binding and altered cytoplasmic localization. *Biochemical J.* **430**, 405–413 (2010).
- Wang, X. et al. Understanding LRRK2 kinase activity in preclinical models and human subjects through quantitative analysis of LRRK2 and pT73 Rab10. *Sci. Rep.* **11**, 12900 (2021).
- Reynolds, A., Doggett, E. A., Riddle, S. M., Lebakken, C. S. & Nichols, R. J. LRRK2 kinase activity and biology are not uniformly predicted by its autophosphorylation and cellular phosphorylation site status. *Front Mol. Neurosci.* **7**, 54 (2014).
- Fan, Y. et al. Interrogating Parkinson's disease LRRK2 kinase pathway activity by assessing Rab10 phosphorylation in human neutrophils. *Biochemical J.* **475**, 23–44 (2018).
- Atashrazm, F. et al. LRRK2-mediated Rab10 phosphorylation in immune cells from Parkinson's disease patients. *Mov. Disord.* **34**, 406–415 (2019).
- Fan, Y. et al. R1441G but not G2019S mutation enhances LRRK2 mediated Rab10 phosphorylation in human peripheral blood neutrophils. *Acta Neuropathol.* **142**, 475–494 (2021).
- Iannotta, L. et al. Divergent effects of G2019S and R1441C LRRK2 mutations on LRRK2 and Rab10 phosphorylations in mouse tissues. *Cells* **9**, <https://doi.org/10.3390/cells9112344> (2020).
- Schmidt, S. H. et al. Conformation and dynamics of the kinase domain drive subcellular location and activation of LRRK2. *Proc. of the Natl. Acad. of Sci. of the USA* **118**, <https://doi.org/10.1073/pnas.2100844118> (2021).
- Karayel, O. et al. Accurate MS-based Rab10 phosphorylation stoichiometry determination as readout for LRRK2 activity in Parkinson's disease. *Mol. Cell Proteom.* **19**, 1546–1560 (2020).
- Cook, D. A. et al. LRRK2 levels in immune cells are increased in Parkinson's disease. *npj Parkinson's Dis.* **3**, 11 (2017).
- Berndsen, K. et al. PPM1H phosphatase counteracts LRRK2 signaling by selectively dephosphorylating Rab proteins. *Elife* **8**, e50416 (2019).
- Liu, Z. et al. LRRK2 phosphorylates membrane-bound Rabs and is activated by GTP-bound Rab7L1 to promote recruitment to the trans-Golgi network. *Hum. Mol. Genet.* **27**, 385–395 (2018).
- Purlyte, E. et al. Rab29 activation of the Parkinson's disease-associated LRRK2 kinase. *The EMBO journal* **38**, <https://doi.org/10.15252/embj.2018101237> (2019).
- Rideout, H. J. et al. The current state-of-the art of LRRK2-based biomarker assay development in Parkinson's disease. *Front Neurosci.* **14**, 865 (2020).

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AUTHOR CONTRIBUTIONS

H.J.R. conceived the study and wrote the manuscript. L.P.V., P.E.V., M.K., & L.D. performed experiments and analyzed the data. A.S., P.E.V., and C.K. performed the clinic visits and collected samples. D.P., A.P., L.S., and R.N.A. supervised the cohorts and patient visits.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Review

The Future of Targeted Gene-Based Treatment Strategies and Biomarkers in Parkinson's Disease

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Abstract: Biomarkers and disease-modifying therapies are both urgent unmet medical needs in the treatment of Parkinson's disease (PD) and must be developed concurrently because of their interdependent relationship: biomarkers for the early detection of disease (i.e., prior to overt neurodegeneration) are necessary in order for patients to receive maximal therapeutic benefit and vice versa; disease-modifying therapies must become available for patients whose potential for disease diagnosis and prognosis can be predicted with biomarkers. This review provides an overview of the milestones achieved to date in the therapeutic strategy development of disease-modifying therapies and biomarkers for PD, with a focus on the most common and advanced genetically linked targets alpha-synuclein (*SNCA*), leucine-rich repeat kinase-2 (*LRRK2*) and glucocerebrosidase (*GBA1*). Furthermore, we discuss the convergence of the different pathways and the importance of patient stratification and how these advances may apply more broadly to idiopathic PD. The heterogeneity of PD poses a challenge for therapeutic and biomarker development, however, the one gene- one target approach has brought us closer than ever before to an unprecedented number of clinical trials and biomarker advancements.

Keywords: alpha-synuclein; LRRK2; glucocerebrosidase; GBA; biomarker; disease-modifying; Parkinson's disease

1. Introduction

More than 6 million people worldwide are living with Parkinson's disease (PD) and it is estimated that this number will double by the year 2040, making PD the fastest growing neurodegenerative disease [1]. Currently, there is neither a clinical biomarker for early detection, diagnosis or prognosis, nor a cure or disease-modifying treatment available for PD. Thus, it becomes evident that biomarkers and disease-modifying therapies are both urgent unmet medical needs in the field and must be developed concurrently because of the paradox: what good are biomarkers for early detection of disease when no disease-modifying therapies are available, or what good are disease-modifying therapies if there are no biomarkers available to detect the disease early enough, i.e., prior to overt neurodegeneration, so patients can have maximal benefit? Another part of the challenge in developing biomarkers and drugs aiming to modify disease is that the pathophysiology of PD is heterogeneous, multifactorial and early stages of the disease have only recently been studied.

PD is a neurodegenerative disorder characterized by the loss of dopaminergic neurons of the substantia nigra pars compacta and cytoplasmic alpha-synuclein (AS)-rich inclusions termed Lewy bodies (LBs). The hallmark motor symptoms of PD are bradykinesia, rigidity, resting tremor, and postural and gait impairment. PD has also a constellation of non-motor symptoms, including depression, anxiety, psychosis, cognitive impairment, autonomic dysfunction, and sleep disorders,

which often precede the motor features by more than a decade [2]. Though the etiology of PD is unknown, ample evidence points towards complex interactions of genetic variants with environmental risk factors [3], with the majority of cases being idiopathic and familial cases accounting for approximately 5–10% of all cases. More than 20 disease-causing gene loci have been associated with PD and 90 independent risk-associated variants based on genome-wide association studies and next generation sequencing [4], many of unknown significance, may potentially play a role in idiopathic PD (iPD) [5]. The α -synuclein (AS) gene (*SNCA*) was the first gene to ever be associated with the development of PD in 1997 [6]. Following this discovery, many more genes have been linked to the disease, including parkin (*PARK2*), UCH-L1 (*PARK5*), PINK1 (*PARK6*), DJ-1 (*PARK7*), leucine-rich repeat kinase-2 (*LRRK2*; *PARK8*), and ATP13A2 (*PARK9*), as well as the risk genes *GBA1*, *VPS35*, *EIF4G1*, and *PARK16*, which have been linked to PD according to family-based linkage analysis. AS and LRRK2 are associated with autosomal dominant PD, while mutations in parkin, PINK1, DJ-1, and ATP13A2 are linked to autosomal recessive PD. LRRK2 mutations are the most common genetic cause of both familial and iPD and the G2019S mutation is the most common, accounting for 5–6% of familial PD and 1–2% of iPD [7]. Heterozygous mutations in the gene encoding β -glucocerebrosidase (*GBA1*) are considered the greatest genetic risk factor for developing PD (5–10% cases) [8,9].

Male sex is associated with higher incidence and prevalence, earlier disease onset, more severe motor symptoms and progression, and more frequent cognitive decline [10]. The interaction between sex and genetics is complex and understudied. Plasma concentrations of AS (see below) are decreased in males with advanced PD, but not females; and this reduction in plasma AS was associated with greater cognitive impairments and sleep disorders [11]. Interestingly, some studies have found a higher prevalence of LRRK2 mutations among women than among men [12]. LRRK2 mutations also show sex-specific phenotypic effects, including milder motor symptoms and higher cognitive function, a lower incidence of REM sleep behavior disorder (RBD) and worse thermoregulation scores in men with LRRK2-G2019S mutation compared to men with iPD, whereas women with LRRK2-G2019S had worse motor complications than women with iPD [13]. In addition, among the patients with LRRK2 PD, women had worse motor complications but better olfaction than men. Our own biomarker study in iPD and G2019S mutation carriers identified several key sex differences with respect to LRRK2 status. First, we have found overall higher expression levels of LRRK2 in PBMCs from males compared to females, and secondly, the elevated in vitro kinase activity was only evident in PBMCs from male subjects [14]. With respect to *GBA1*, there appears to be an increased risk of neuropsychiatric comorbidities, such as anxiety and depression, in men but not women with *GBA1* mutations [15], suggesting a sex-genotype interplay in PD symptom manifestation.

With respect to biomarkers, we presently focus on biofluid studies (i.e., cerebrospinal fluid (CSF), blood cells and urine). However, other methods including neuroimaging and electrophysiology, along with clinical assessment, provide a vital source of diagnostic and prognostic information and are an equally active area of research. Magnetic resonance imaging (MRI) focusing on structural brain differences looks promising in distinguishing between iPD and other parkinsonian phenotypes (e.g., MSA) [16]. By looking at the functional level, changes in circulating oxygen levels may also differentiate iPD from a healthy state [17]. These abnormalities can occur prior to disease onset; hence, they may also serve as a prognostic tool [18]. Positron emission tomography (PET) or single photon emission computed tomography (SPECT) measure differences at the molecular level by looking, among others, at the dopamine transporter (DAT), approved by the European Medicines Agency as a diagnostic tool [17], dopamine release [17] or glucose metabolism [17,19] using radiolabelled compounds that cross the BBB (e.g., 18F-DOPA, 18F-FDG). In addition, transcranial magnetic stimulation (TMS) provides information about cortical function deficits, a feature commonly observed in movement disorders such as PD [20], and electrophysiology can be used for disease monitoring by measuring neuronal activity directly by wave quantification with the aid of scalp electrodes [21]. To what extent these methods can be used reliably as biomarkers is still questionable and, as PD is a multidimensional disease, a combination of multiple approaches may be essential.

This review provides an overview of the rationale for and progress in the development of novel biomarkers and disease-modifying treatments for PD. This review focuses on the three most studied genetic pathways—AS, LRRK2 and GBA—that are the most advanced in terms of preclinical proof-of-concept studies, therapeutic development and clinical trial success to date (see Figure 1 for a timeline of milestones, Figure 2 for a summary of drug targets). Finally, we will focus on the three most studied gene targets, alpha-synuclein (AS), glucocerebrosidase (*GBA1*), and leucine-rich repeat kinase-2 (*LRRK2*), but we will also discuss the overlapping commonalities of the three pathways and the importance of patient stratification and how this knowledge pertains to iPD. This is not meant to be an exhaustive literature search but rather to highlight the urgency, bridge the current trends in translational and clinical research in the discovery and development of biomarkers and disease-modifying therapies for PD and, finally, provide the impetus for stimulating, innovative and game-changing research.

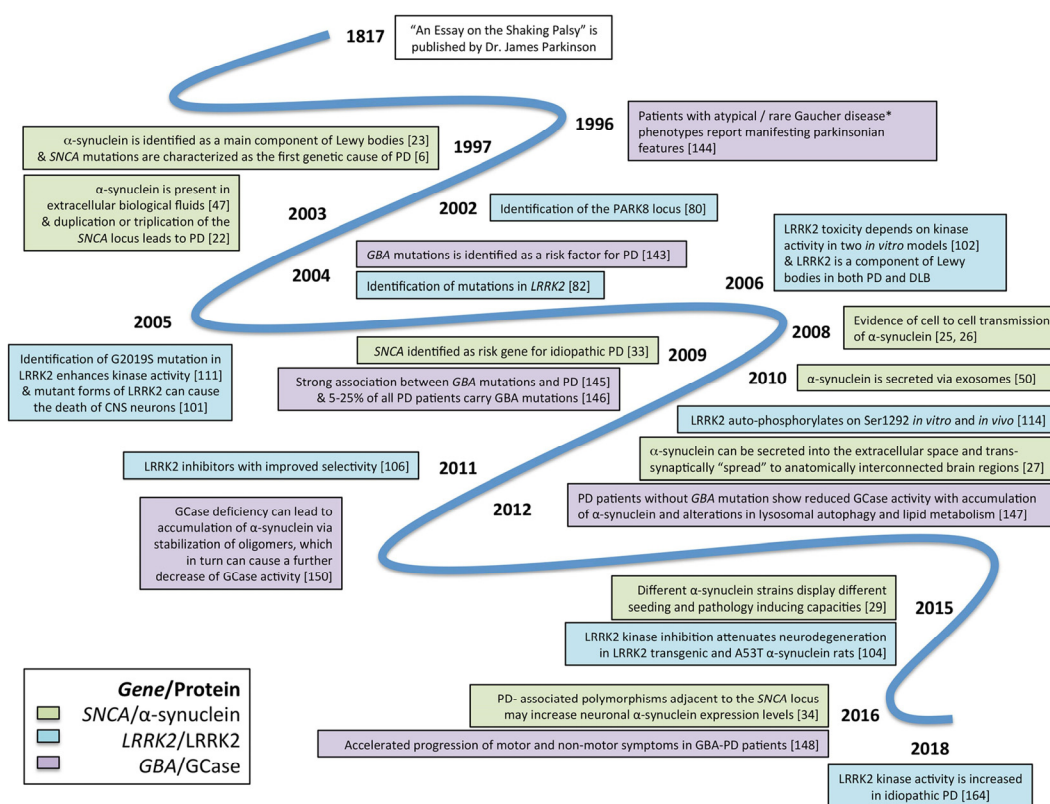


Figure 1. Timeline of milestone discoveries related to the three main genetic PD targets; α-synuclein, LRRK2 and GBA. DLB, Dementia with Lewy bodies; GBA, P-Glucocerebrosidase (GCase) gene; LRRK2, Leucine—rich repeat kinase 2; PD, Parkinson’s disease; SNCA, α-synuclein gene; * Gaucher disease is a lysosomal storage disorder caused by biallelic GBA mutations.

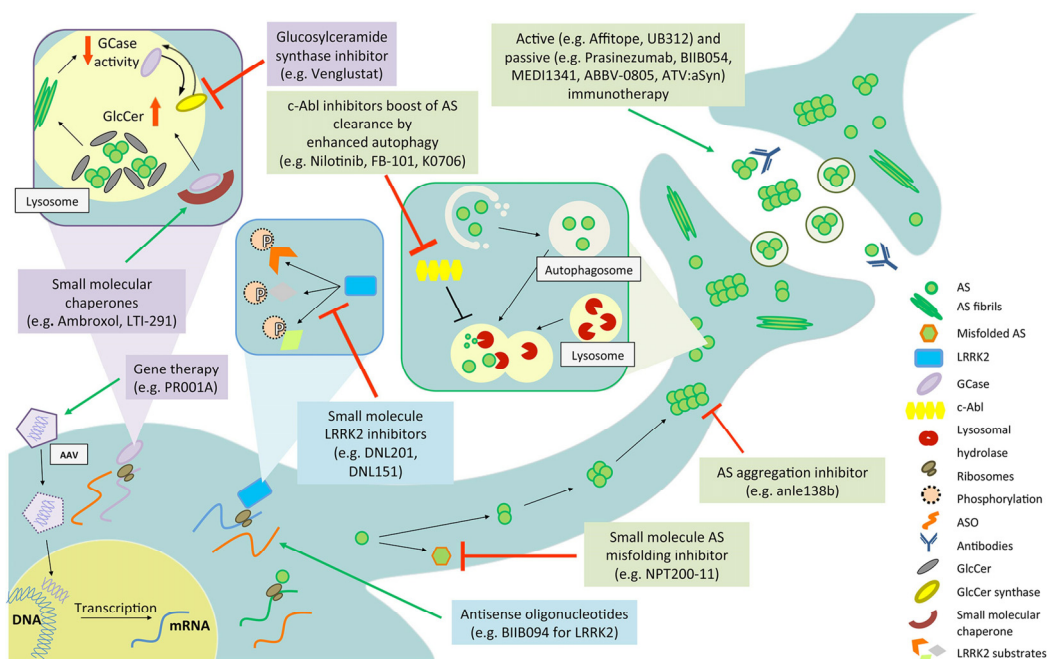


Figure 2. Mechanisms of action of the potential therapeutic compounds currently in clinical trials. As, Alpha-synuclein; ASO, Anti-sense oligonucleotides; GlcCer, Glucosylceramide; LRRK2, Leucine-rich repeat kinase 2.

2. Literature Search Criteria

The literature review encompassing therapeutic strategies targeting these specific proteins used expanded search criteria to include the following:

- “synuclein AND antisense”; “synuclein AND passive/active immunization”.
- “LRRK2 AND kinase AND inhibitor”; “LRRK2 AND antisense”.
- “GBA/GCase/glucocerebrosidase AND therapeutic”; “GBA/GCase/glucocerebrosidase AND ambroxol”.

For the purposes of the literature review with respect to biomarkers, examples of our search terms include the following:

- “synuclein AND CSF”; “synuclein AND plasma/serum”; “synuclein AND saliva”.
- “LRRK2 AND CSF”; “LRRK2 AND PBMC/monocyte/neutrophil”; “LRRK2 AND urine”.
- “GBA AND biomarker”; “GCase AND biomarker”; “glucocerebrosidase AND biomarker”; “GBA/GCase/glucocerebrosidase AND activity”.

We used both the PubMed as well as clinicaltrials.gov databases for our review, and did not explicitly exclude publications based on publication date, in order to provide some historical context to the genetic bases of PD pathogenesis, and thus therapeutic discovery.

3. Therapies Targeting Alpha-Synuclein

Alpha-synuclein (AS) is a presynaptic protein and arguably has the most central role in PD pathogenesis as: 1) multiplications and mutations of the AS gene (*SNCA*) locus cause familial PD [6,22], 2) aggregated AS is a major constituent of LBs and Lewy neurites, the pathological hallmark in AS-related PD and most idiopathic cases [23] and 3) AS can be secreted into the extracellular space and trans-synaptically “spread” to anatomically interconnected brain regions [24–27]. In addition, genetic, viral and pathogenic AS-inoculated animal models have demonstrated that overexpression of wildtype, mutant and fibrillary AS in neurons is associated with AS aggregation and nigral neurodegeneration [28], with different AS conformations leading to distinct histopathological and

behavioural phenotypes [29]. Finally, polymorphisms that occur within specific regions of the *SNCA* gene increase the overall risk of PD by either increasing the production or slowing the clearance of AS [30–34]. Altogether, AS appears to be linked with both iPD and familial forms of PD. Based on this evidence, several therapeutic strategies currently being pursued include reducing AS load via: (1) reduction in gene and protein expression, (2) restoration of AS proteostasis or 3) reduction in AS propagation. There are currently 10 active clinical studies (www.clinicaltrials.gov), in which AS is the primary drug target (summarized in Table 1).

Table 1. α -synuclein therapeutics.

Compound	Mechanism of Action	Clinical Indication	Patient Group	Sponsors	Clinical Phase	Time Period (Ongoing)	Clinical Trial
NPT200-11 (UCB0599)	Small molecule α -synuclein misfolding inhibitor	PD	Healthy volunteers	UCB, Neuropore Therapies	1: Safety, Tolerability, PK	07/2015–02/2016	NCT02606682
anle138b	α -synuclein aggregation inhibitor	PD	Healthy volunteers	MODAG GmbH Quotient Sciences Aptuit (Verona) Srl	1: Safety, tolerability, blood levels	12/2019	NCT04208152
Glyceryl phenylbutyrate *	α -synuclein clearance from brain to blood	PD	iPD	University of Colorado, Denver	1: Efficacy	01/2014	NCT02046434
Nilotinib *	Abl inhibitor; autophagy enhancer; boosts α -synuclein clearance	PD +/- dementia	PD +/- dementia iPD	Georgetown University Northwestern University	2: tolerability, safety	01/2017–10/2017	NCT02954978 NCT03205488
K0706 (SCC-138)	Abl tyrosine kinase inhibitor	PD	Clinically probable PD	Sun Pharma Advanced Research Company (SPARC)	2: safety, efficacy	02/2019-	PROSEEK NCT03655236
FB-101	c-Abl inhibitor; autophagy enhancer; boosts α -synuclein clearance	PD	Healthy volunteers	1 st Biotherapeutics	1: safety, tolerability, PK	03/2019-	NCT04165837
Prasinezumab (PRX002/RO7046015)	α -synuclein passive immunotherapy	PD	iPD	Roche, Prothena	2: MDS-UPDRS changes	06/2017	PASADENA NCT03100149
BIIB054 (cinpanemab)	Human—derived antibodies; targets aggregated α -synuclein	PD	PD	Neurimmune, Biogen	2: Safety, PK/PD	01/2018	SPARK NCT03318523
MEDI1341	α -synuclein passive immunotherapy	PD	Healthy volunteers	AstraZeneca, Takeda	1: Safety, tolerability, PK/PD	10/2017	NCT03272165
ABBV-0805	α -synuclein passive immunotherapy	PD	iPD	AbbVie	1: Safety, tolerability	03/2020-	NCT04127695
ATV:aSyn	α -synuclein passive immunotherapy	PD	n/a	Denali Therapeutics	IND	-	-
Affitope PD01A/PD03A	α -synuclein active immunotherapy	PD	Early PD, iPD	Affiris	1: Immunogenicity, AEs	02/2016–02/2017	NCT01885494^ NCT01568099^ NCT02216188^

							NCT02267434^ NCT02618941^
UB312	α -synuclein active immunotherapy	PD	Healthy volunteers	United Neuroscience	1: Safety, tolerability, immunogenicity	08/2019-	NCT04075318

Table 1 Alpha-synuclein (AS)-based disease-modifying treatment strategies and their current progress in clinical trials. iPD = idiopathic PD, * Repurposed drugs, ^ completed clinical trial, AEs = adverse effects, PK = pharmacokinetics, PD = pharmacodynamics; Clinical phases: 1 =Phase 1, clinical trials that focus on drug safety typically conducted with healthy volunteers to determine the drug's pharmacokinetics and most frequent and serious adverse events; Phase 2, clinical trials that focus on the drug's effectiveness in patients populations while continuing to study its safety and short-term adverse events.

3.1. AS Expression Reduction

α -synuclein (AS) was the first gene (*SNCA*) to ever be associated with the development of PD [6]. Duplication or triplication of the *SNCA* locus leads to PD [22] and a number of missense mutations have been identified, of which A53T is the most common [6]. PD-associated polymorphisms adjacent to the AS gene locus have recently been identified and may possibly increase neuronal AS levels [34], supporting the use of AS-lowering therapies in idiopathic cases as well. Thus, one inevitable approach, following the therapeutic success of antisense oligonucleotides (ASOs) for spinal muscular atrophy [35] and Huntington disease [36], includes the inhibition of AS expression via targeted ASOs. ASOs are single-stranded RNA oligomers that are complementary to specific sequences within a mRNA molecule. Once bound, the mRNA is digested, typically via RNase H. This approach is currently being pursued at the preclinical level and has demonstrated sufficient AS downregulation in rodents and non-human primates (Ionis pharmaceuticals; [37]).

A fascinating epidemiological study in Norway regarding the long-term use of β 2-adrenoreceptor agonists and antagonists leading to reduced and increased risk of PD, respectively, also experimentally demonstrated that β 2 receptors regulate AS transcription [38]. These findings led to hope for repurposing β 2-adrenoreceptor agonists as potential disease-modifying treatment of PD, however, more recent epidemiological studies have failed to demonstrate these correlations and consider the relative protection with β 2-adrenoreceptor agonists to be an indirect effect of smoking (an established reduced risk factor for PD) and the higher risk with β 2-adrenoreceptor antagonists to reverse causation, i.e., PD symptoms trigger their use rather than their use triggering PD [39,40]. Thus, further studies are warranted to explore the potential for pharmacologically modulating AS expression via β 2 receptors.

3.2. Restoration of AS Proteostasis

The presence of AS aggregates in LB inclusions of diseased brains suggests dysfunctional proteostasis due to numerous factors including gene mutations, post-translational modifications, external toxins, neuroinflammation, oxidative stress and age-related dysfunction of proteolysis [41]. These aberrant forms of AS, especially the soluble oligomeric forms, were identified as the main culprit of cell death [42]. Thus, by restoring proteostasis by either inhibiting protein misfolding and aggregation or enhancing AS clearance, AS pathology could potentially be diminished, possibly slowing or halting disease progression. Based on this evidence, NPT200-11 (UCB0599, UCB/Neuropore Therapies), a small molecule AS misfolding inhibitor has shown promise in improving AS pathology and motor function in vivo [43] and completed phase I trials for tolerability in 2016 (NCT02606682). Anle18b (MODAG GmbH), an AS aggregation inhibitor is currently being developed for the synucleinopathy Multiple System Atrophy, with potential for future use in PD, is now in a Phase 1 trial for safety and tolerability (NCT04208152). Alternatively, glyceryl phenylbutyrate, an FDA approved drug for urea cycle disorders, is being tested in a phase I trial (sponsored by the University of Colorado) to assess whether or not it can boost the clearance of AS from the brain into the bloodstream (NCT02046434). Another approach includes the enhancement of autophagic degradation of AS with c-Abl tyrosine kinase inhibitors [44,45]. The anti-cancer drug, nilotinib, an inhibitor of the tyrosine kinase c-Abl, is being tested in phase 2 trials for tolerability, safety, pharmacokinetics and motor symptom improvement (sponsored by Georgetown University, NCT02954978; and Northwestern University, NCT03205488), the first of which demonstrated safety, increased CSF levels of the dopamine metabolite homovanillic acid, and a reduction of oligomeric AS [45]. However, the second study, in press, found no evidence of symptomatic benefit, low CSF drug exposure and no changes in CSF dopamine metabolite levels [46]. Furthermore, K0706 (SCC-138) is being tested for safety and efficacy in a phase 2 trial (Sun Pharma Advanced Research Company, NCT03655236) and FB-101 entered phase I trials last year to test safety, tolerability and pharmacokinetics (1ST Biotherapeutics, NCT04165837).

3.3. *Trans-Synaptic Transmission Reduction*

AS can be secreted into the extracellular space [47–50], taken up by neighboring neurons, recruiting endogenous AS and perpetuating disease spreading to interconnected brain regions [51]. Thus, the most advanced clinical programs targeting AS include capturing extracellular AS with either passive immunotherapy, i.e., antibodies specific to AS or active immunotherapy, i.e., the administration of modified AS to induce the production of endogenous antibodies. Clinical trials using the passive immunotherapy approach include prasinumab (PRX002/ RO7046015; Roche, Prothena), which is in phase II (NCT03100149), the PASADENA trial for evaluation of MDS-UPDRS changes, after results demonstrated safety and proof of target engagement as a robust reduction in free serum AS and dose-dependent increases in the antibody in cerebrospinal fluid [52]. BIIB054 (cinpanemab, Neurimmune/Biogen) is also in phase II to assess safety and the pharmacokinetic/dynamic profile and both appear to be more specific for aggregated AS (NCT03318523) [53]. MEDI1341 (AstraZeneca, Takeda) is in Phase I for safety, tolerability and pharmacokinetic/dynamic profile (NCT03272165) and ABBV-0805 (AbbVie) is entering Phase I (March 2020) for safety and tolerability (NCT04127695) while Denali Therapeutics is pursuing an investigational new drug (IND) application for ATV:aSyn. With respect to active immunotherapies, Affitope (PD01A/PD03A, Affiris) has completed phase 1 trials for safety, tolerability, immunogenicity and clinical activity for PD as well as MSA (NCT02618941) and UB312 (United Neurosciences) for safety, tolerability and immunogenicity (NCT04075318). These therapies have great potential if indeed, by reducing extracellular AS, the load of hypothetically toxic AS aggregates and further propagation of AS to anatomically interconnected brain regions is diminished.

We anticipate this year that the results of several ongoing clinical trials will demonstrate which, if any, AS-targeted therapeutic strategies stand a chance of slowing or halting the progression of PD. Either way, it is vital that we continue to build on our knowledge of AS's physiological role to better understand its role in PD pathogenesis, especially considering the consequences of reducing AS synthesis. Moreover, the presence of LB pathology does not necessarily prove AS's toxic role and it is possible that AS aggregation may be a mere epiphenomenon or protective mechanism involving the sequestration of misfolded protein [54]. The inherent difficulty in elucidating the significance of LBs also highlights the importance of focusing efforts on identification of early biomarkers in the prodromal stage of disease to achieve the greatest future benefit from disease-modifying therapies. Finally, the ultimate effectiveness and range of therapeutic benefit of targeting different AS forms and conformational states remains to be known as the toxic AS species has not been established. The same applies to small antibodies that only target extracellular AS: will the achieved reduction in AS load be sufficient to slow or halt disease progression? These questions necessitate rigorous preclinical assessment in multiple *in vivo* models (both genetic and environmental) and direct measures of extracellular AS levels to help de-risk clinical trials, especially when generalizing results to idiopathic cases.

3.4. *AS-Targeted Biomarkers*

No biomarker to date has reached the desired sensitivity/specificity in order to be used in clinical practice and aid clinical trials. A major challenge in the biomarker field is that other neurological disorders (MSA, Alzheimer's disease, etc.) share similar pathologies with PD and many of these protein changes overlap [55,56]. As a result, it is important to develop robust assays of high sensitivity and specificity for differential diagnosis. AS is present in different biofluids [57]. As it is a protein readily expressed in the brain, the most promising biofluid source is cerebrospinal fluid (CSF), which represents a snapshot of the central nervous system environment [57]. However, blood, saliva and peripheral tissues are also good candidates and may be preferred as more accessible and less invasive [58]. Pathology has mostly been associated with aggregate inclusions in LBs, where ~90% of the protein is Ser129 phosphorylated [59]. Thus, identification of any differences in the levels of these species and the pathways involved in disease is critical in order to develop biomarkers for the prognosis, diagnosis and monitoring of PD (see Summary Box 1).

Box 1: Overview of AS-targeted biomarkers.*CSF*

- Most studies report that PD patients have lower levels of total AS compared to healthy controls [60,61] but increased levels [62] or absence of changes in the levels of total AS have also been reported [63].
- Early-diagnosed treatment-free PD patients have a decline in total AS levels which can successfully predict cognitive decline but not motor defects [64]. Medication does not contribute to this decline in total AS levels [62,65].
- PD patients are reported to have increased levels of non-normalised oligomeric AS [56,62] and a proportional relationship was observed regarding oligomeric/total AS ratio to motor symptom severity [62].
- The ratio of phosphorylated AS (pS129) to total AS is found elevated in PD patients compared to healthy controls [56] while a different longitudinal study reports a decrease of pS129 [62].

Blood

- The results from total [66], truncated and oligomeric AS forms [48,67] isolation from plasma/serum are generally inconclusive.
- Elevated phosphorylated AS forms remain stable as the disease progresses while total AS levels are shown to increase during the years [68].
- Exosomes of neuronal origin, circulating in the plasma of PD patients, show higher levels of total AS compared to healthy controls [69] correlating weakly with the severity of motor symptoms [69].

Saliva

- Total AS is lower and negatively correlated with the oligomeric form in PD patients compared to healthy controls [70].
- Findings of pS129/total AS ratio being higher in neuronal exosomes of PD patients versus control samples correlating with poor motor assessment [71], however, this was not reproducible in a larger cohort [70,72].
- PD patients show elevated oligomeric AS or oligomeric/total AS ratio versus control samples [70,71] not associated with disease severity [72].

Gut mucosa

- PD patients show higher levels of intestinal AS compared to healthy controls [73,74].
- Detection of AS conformational variants considered pathological is inadequate for diagnosis or prediction of PD [75].

3.5. CSF

Overall, there is a consensus that total AS levels are lower in CSF coming from PD patients compared to healthy controls [60,61]. This reduction may be explained by the degeneration of neurons/synaptic loss, increased inclusion of AS in LBs or by defects in protein's gene expression, which are all hallmarks of the disease [61,63]. AS levels are not only lower in CSF but can also differentiate PD patients from healthy controls [76]. Nevertheless, no differences in AS levels have also been reported [63]. This discrepancy may be explained by the smaller cohort in the latter study, by differences in the antibody affinities used or by possible CSF contamination with AS from blood that can contribute to false-positive measurements [76]. On the other hand, total AS alone is likely insufficient for PD diagnosis, characterised by lack of specificity and, instead, total levels should be examined as a ratio to ubiquitously expressed control proteins [77]. Considering that toxicity linked to PD pathology is mostly associated with truncated forms of AS, current research has shifted attention to examining each of these species separately and in relation to total levels. Immunoprecipitation and mass spectrometry experiments have demonstrated the presence of these modified forms in CSF [78,79]. For example, despite the lower levels of total protein observed, pS129/total ratio appears to be elevated in PD [56], and the same is true for non-normalised oligomeric protein [55]. In this regard, different outcomes can arise depending on the type of species under investigation, thus, ideally, multiple biomarkers should be assessed simultaneously.

As PD is a progressive disorder, biomarkers for severity progression are needed. The correlation of protein levels with motor deficits, cognitive decline and disease severity has proven to date [55,80]. Some promising results were obtained from a longitudinal study monitoring patients for 2–8 years [64]. Total AS CSF levels were low in early-diagnosed, treatment-free PD, dictating that this change happens early in the disease and is irrespective of drug use [64]. It was also shown that total α -synuclein was diminished during the first 2 years and this could successfully predict cognitive decline but not motor defects [64]. Two cross-sectional studies in drug-naïve patients further confirmed that medication does not contribute to this decline in total α -synuclein levels [65,81]. On the contrary, in a second longitudinal study total levels as well as oligomeric AS increased whereas pS129 decreased over the 2-year course [62]. A proportional relationship was also observed regarding oligomeric/total AS ratio to motor symptom severity [62]. These controversial findings need further elucidation as to whether any of these species can be reliably used as a biomarker target in PD. Additionally, a gap in the experimental field is the lack of routine assays available to detect the aggregated protein form that is greatly implicated in pathology. Real-time quaking-induced conversion (RT-QuIC) is a new, promising assay providing an alternative way of detecting seeding species in CSF by amplification, characterised by very high specificity and sensitivity [82–84].

3.6. Blood

Although blood is an easily accessible biofluid source, results from AS isolation from plasma/serum are generally inconclusive for biomarker purposes [66]. According to an early study, levels of total AS are lower in PD patients compared to healthy controls and this is more apparent in early-onset versus late-onset PD [85]. A later study, however, showed elevated plasma AS positively correlating with cognitive decline [66]. Other groups failed to report such differences and more studies are needed to clarify these discrepancies in the literature [58,86]. Controversial findings also exist for the detection of truncated AS and oligomeric forms in plasma as well [49,67]. Regarding pS129 phosphorylated forms, elevated levels remained stable for up to 20 years after diagnosis and total levels were shown to increase during the years, asserting that total α -synuclein could potentially be used as a marker for disease progression [68].

Radiolabelled AS administration to mice brains has demonstrated that it can be readily transported to blood circulation, probably via exosome release through the blood brain barrier (BBB) [69,87]. Exosomes are small vesicles (~30–150 nm) of the endocytic pathway that can move from cells to the periphery, carrying the intracellular cargo into the body's circulation [88]. Hence, it is believed

that exosomes originating from the brain circulate in plasma, and upon isolation their content will be reflective of the neuronal state [87]. There are generally more consistent findings based on this approach rather than measuring α -synuclein freely floating in plasma; however, neuronal exosome isolation can be challenging. Early evidence came from immunoprecipitation studies against L1CAM neuronal exosome marker [69]; their presence was confirmed by Immuno-EM [69], however, only a small fraction of injected AS was enclosed in exosomes while the rest was detected as a free form in the plasma [69]. In contrast to lower levels of AS in CSF exosomes of PD patients, plasma exosomes had higher protein levels either alone (in exosomes only) or when normalised to total protein in whole plasma [69]. This increase in plasma may also explain the lower levels in CSF as a result of exosomal leakage to the periphery, probably serving as an alternative clearing mechanism of toxic species, since cell degradation pathways are defective in PD [89]. There was a weak correlation between high AS levels and severity of motor symptoms, while there was no such link with CSF samples, suggesting that plasma may be more useful as a biomarker for disease progression [69,90].

3.7. Saliva

The use of saliva in biomarker studies is a current trend as there is increasing evidence of AS being a major component [91,92]. From a recent pilot study, it was shown that exosomes isolated from saliva by ultracentrifugation are of a neuronal origin and express α -synuclein [71]. They demonstrated by immunoblotting that pS129/total α -synuclein ratio is higher in PD L1CAM-positive exosomes versus control samples [71]. Disease progression could not be related to these high levels, attributed to the small number of participants, but there was a correlation with poor motor assessment [71]. In a larger cohort, however, total protein, pS129 or pS129/total ratio were stable, whereas oligomeric or oligomeric/total protein was elevated [70,72]. No association with disease severity and high oligomeric levels was found [72]. Total protein was lower and negatively correlated with the oligomeric form [70].

3.8. Gut Mucosa

AS measurements in the gastrointestinal tract (GIT) have also been proposed as a potential PD biomarker based mainly on the Braak hypothesis that states that the origins of PD pathology may begin in the GIT [93] and subsequently reaches the CNS via the dorsal motor nucleus of the vagus nerve [94]. However, despite the lack of complexity characterizing *in vivo* biopsies, the heterogeneity of the GI tract adds to the variability rising from different methodological approaches (i.e., antibodies, antigen retrieval methods, techniques) [75]. Transgenic animal models overexpressing AS show higher levels of intestinal AS [73,74] corroborating what was previously reported in PD patients [95,96]. However, despite earlier work delineating the use of GI AS as a predictive biomarker [95–97], more recently, the detection of pathological AS conformational variants has been considered inadequate for the diagnosis or prediction of PD, in spite of using different methods for optimal specificity and sensitivity [75]. Altogether, the detection of specific AS species (i.e., phosphorylated and oligomeric AS) when compared to total AS levels, and samples most representative of LB origin (i.e., neuronal-derived plasma exosomes and saliva), appear to be the most promising AS-related PD biomarkers.

4. Therapies Targeting LRRK2

Quite soon after the cloning and identification of the LRRK2 gene in the PARK8 locus on chromosome 12 [98–100], it was established that mutant forms of the protein that segregate with the disease could cause the death of CNS neurons [101]. Subsequent studies went further, and demonstrated, using both genetic and pharmacological approaches, that this neuronal death was kinase-dependent (e.g., [102,103]). While it remains to be demonstrated what the relevant substrate(s) are, and in which specific tissues (e.g., cell autonomous vs. non-cell autonomous) they are found, this kinase dependency was also demonstrated in viral *in vivo* models [104,105]. With these findings in hand, considerable resources, both private and academic, were dedicated to the search and

characterization of potent and selective small molecule inhibitors of LRRK2 kinase activity, with the goal of identifying novel therapeutic candidates. First-generation LRRK2 kinase inhibitors (e.g., IN-1, CZC-25146; [106,107]) showed decent affinity and moderate selectivity, however even at lower concentrations still exhibited off-target effects and were unable to cross the blood-brain-barrier (BBB). Nevertheless, they served, and are still used, as useful “tool inhibitors” in *in vitro* and cellular studies of LRRK2 activity. Subsequent generations of inhibitors exhibited greatly improved potency, selectivity, and the ability to cross the BBB.

In 2012, Genentech, with support from the MJFF-LRRK2 Safety Initiative, published the findings of a pre-clinical safety trial of two structurally distinct LRRK2 kinase inhibitors, GNE-7915 and GNE-0877, in rodents and non-human primates [108]. Both compounds showed very high selectivity for LRRK2 among several kinase libraries, and exhibited a low nM-range IC₅₀'s, in *in vitro* assays [108]. Both compounds are able to cross the BBB, and induced robust de-phosphorylation of pS935-LRRK2 (a marker of LRRK2 inhibition). Interestingly, while, in the brain, total LRRK2 levels were unaffected by 15 days of dosing with two doses of each compound, in the kidney, which expresses very high levels of LRRK2, total LRRK2 levels were significantly reduced [108]. This observation was markedly clear in the mouse, however, in the non-human primate model, brain levels of total LRRK2 trended lower as well. The explanation for this difference is unclear, however, it is noteworthy that a specific fragment of LRRK2, corresponding either to a translated splice variant or a proteolytic processing of full-length LRRK2, is observed selectively in kidney extracts. With relevance to their use in human clinical trials, however, exposure to both compounds induced an accumulation of lysosome-related lamellar bodies in type-II pneumocytes in non-human primates, phenocopying an observation in LRRK2-deficient mice [109]. Follow up safety studies in non-human primates indicated that the pathological changes observed in type II pneumocytes in treated animals were reversible and, importantly, did not lead to appreciable functional deficits in pulmonary function [110].

In 2018, Denali Therapeutics Inc. launched clinical trials of two distinct LRRK2 kinase inhibitors, DNL151 and DNL201, at centres in The Netherlands (NCT04056689) and the United States (NCT03710707), respectively. The first Phase I study was designed to assess safety, tolerability, pharmacokinetics, and pharmacodynamics in healthy volunteers using two doses of the DNL201 kinase inhibitor. In addition to evaluating the metabolism and stability of the compound, key outcome measures included biochemical indicators of target engagement, i.e., that LRRK2 kinase activity was in fact reduced in subjects receiving each dose of the test compound. We discuss the currently available LRRK2-targeted biomarkers in more detail below; however, in this clinical study, phosphorylation of LRRK2 at the Ser935 residue as well as phosphorylation of the LRRK2 substrate Rab10 at the Thr73 residue, was assessed in several biofluids (e.g., whole blood or PBMCs, CSF). Initial findings released by the company for both compounds indicated that targets for safety as well as target engagement were both met with both compounds. It is expected that clinical evaluation with one of the compounds will proceed to the next stage, with recruitment of PD patients both with and without the common G2019S mutation [111]. A potential obstacle might arise in finding adequate numbers of mutation carriers in order to have a sufficiently powered study to evaluate the efficacy of these compounds. This issue will potentially create a “bottleneck” as additional investigational compounds enter the playing field, competing for study participants. Nevertheless, the early, encouraging results of these compounds can only serve to embolden the approach of targeting LRRK2 kinase activity as a potential disease-modifying therapy.

Alternative approaches to block the kinase function of LRRK2 include ASOs, where the aim is to reduce the expression of LRRK2, thereby reducing the net kinase activity in the target cells. ASOs are single-stranded RNA oligomers that are complementary to specific sequences within a mRNA molecule. Once bound, the mRNA is digested typically via RNase H. An example of such an approach is the compound BIIB094/ION859, developed jointly by Biogen and Ionis pharmaceuticals. There is an actively recruiting Phase I safety trial currently underway assessing single and multiple ascending doses (NCT03976349). In a recent collaborative study between Ionis Pharmaceuticals and researchers at the University of Alabama, LRRK2 ASO's proved to be effective in reducing AS pathology in several models of iPD. First, in long-term cultures of primary hippocampal neurons,

two independent ASO's targeting LRRK2 effectively reduced the appearance of pS129-AS inclusions following treatment with AS pre-formed fibrils (PFFs; [112]). Extending this to an in vivo PFF model, the authors reported that both LRRK2 ASO's reduced the abundance of pS129-AS inclusions by approximately 50% in comparison to controls [112]. There are several key findings to highlight from this work. First, this serves as another important piece of evidence indicating that CNS LRRK2 plays a critical role in PD progression, even in models of iPD, not associated with LRRK2 mutations. Secondly, it strengthens the link and further defines the pathway between LRRK2 activity and AS pathology in PD. In other words, as demonstrated in this model, pathological extracellular AS (PFFs) requires LRRK2 activity in order to induce downstream (i.e., in recipient neurons) pathological changes. Finally, this work demonstrates that centrally targeting LRRK2 activity appears to be sufficient in reducing AS pathology, although further work is needed to fully characterize, for example, cross-talk between CNS neurons and peripheral immune cell signalling. A further advantage of this approach is the inherent flexibility to modify the target sequence, thus providing the possibility to target only the mutant allele in heterozygous carriers of LRRK2 mutations. Such an approach has been taken in studies aiming to reduce the expression of mutant huntingtin protein [36] (for a summary of LRRK2 therapeutics, see Table 2).

One note of caution, pertaining to both approaches (small molecule inhibitors of LRRK2 kinase activity, or ASO down-regulation of LRRK2), concerns the uncertainties surrounding the long-term, system-wide inhibition of LRRK2 activity. It is unclear at this point whether the enduring inhibition of LRRK2 activity will negatively impact any vital functions of LRRK2 that are unrelated to its role in PD pathogenesis or progression. Moreover, while it has been clearly demonstrated that the inhibition of LRRK2 kinase activity is effective in pre-clinical models of familial and iPD, the relevant substrate(s) of LRRK2 linked to PD pathogenesis or progression have not been identified yet, highlighting the need for continued work in this area.

Table 2. Leucine-rich repeat kinase 2 (LRRK2) therapeutics.

Compound	Mechanism of Action	Clinical Indication	Patient Group	Sponsors	Clinical Phase	Time Period (Ongoing)	Clinical Trial
DNL151 DNL201	Small molecule LRRK2 inhibitors	PD	PD PD, LRRK2- PD	Denali Therapeutics	1: Safety, tolerability, PK/PD 1b Safety, PD/PK	07/2019– 12/2018	NCT04056689 NCT03710707
BIIB094	LRRK2 antisense oligonucleotides	PD	PD	Biogen, Ionis Pharmaceuticals	1: Safety, tolerability, PK	08/2019–	REASON NCT03976349

Table 2 Leucine-rich repeat kinase 2 (LRRK2)-based disease-modifying treatment strategies and their current progress in clinical trials. PK = pharmacokinetics, PD = pharmacodynamics; Clinical phases: 1 =Phase 1, clinical trials that focus on drug safety typically conducted with healthy volunteers to determine the drug's pharmacokinetics and most frequent and serious adverse events; Phase 2, clinical trials that focus on the drug's effectiveness in patients populations while continuing to study its safety and short-term adverse events.

4.1. LRRK2-Targeted Biomarkers

A common underlying need in clinical studies evaluating LRRK2-based therapies is a quantitative, sensitive, and minimally invasive biomarker. Whether the specific outcome measure is reducing LRRK2 expression levels (e.g., the ASO approach), or reducing its kinase activity (e.g., small molecule inhibitors), reliable biochemical assays of well-defined targets are needed. Many biomarker studies focus on assessing LRRK2 posttranslational modifications and activity, as the pathogenic mutations have been associated with changes in protein phosphorylation levels and its endogenous substrates in in vitro and in vivo experiments [113,114]. If such changes prove to be distinct in

biofluids of PD patients, they could potentially be used for drug target engagement and/or as inclusion/exclusion criteria in clinical trials of LRRK2 inhibitors. Lines of evidence highlight the central role of LRRK2 in the inflammatory response, which is robustly altered in PD [113,115,116]. As a result, considerable interest has arisen in mapping the protein's expression patterns in different immune cell populations present in blood. Attention has also been directed to exosomal isolation from other peripheral biofluids such as CSF and urine, where LRRK2 (and other PD-linked proteins) is present [117–120] (see Summary Box 2).

Box 2: Overview of LRRK2-targeted biomarkers.

Blood

- LRRK2 is highly expressed in monocytes and neutrophils of PD patients [121,122]. Total LRRK2 levels are significantly elevated in CD16⁺ monocytes as well as T-cells and B-cells compared to healthy controls [121].
- Despite the inability to detect changes in total or phosphorylated LRRK2 levels in PBMCs of iPD patients [122–124], G2019S PD patients show reduced pS935 levels compared to iPD patients, G2019S healthy carriers and healthy controls, not correlating with motor symptoms or cognitive decline [125].
- The use of Rab GTPases is generally inconclusive. While the phosphorylation of Rab10 (pRab10) is reported to be LRRK2 dependent [126] there are no significant differences observed in PBMCs and neutrophils between PD patients and controls in pRab10 levels [122,127].

CSF

- No significant changes were observed between G2019S related PD patients and healthy groups in the levels of pS1292 [128].

Urine

- Normalisation of pS1292 levels to total LRRK2 from urinary exosomes can predict PD risk with different levels of accuracy among G2019S carriers with PD, PD non-carriers, sporadic PD patients and healthy participants of both sexes [117–119]. Poor clinical test scores are correlated with heightened pS1292 levels in male PD patients [119].
- Regardless of disease status, total LRRK2 levels are higher in females, though without any significant differences between PD and healthy groups [116–119].
- The levels of LRRK2 expression are higher in the PBMCs of males (14).

4.2. Blood

High expression levels of LRRK2 are evident in monocytes (particularly CD14⁺ and CD16⁺ types), which make up ~1%–20% of peripheral blood mononuclear cells (PBMCs), as well as neutrophils (another type of leukocytes) [121,122]. As shown by flow cytometry, total levels are significantly elevated in CD16⁺ monocytes, T-cells and B-cells in PD individuals compared to healthy controls [121]. In addition, this increase was positively correlated with cytokine release upon inflammatory stimulation in PD [121]. Phosphorylated levels of LRRK2 at pS910, pS935, pS955 and pS973 sites have also been studied in PBMCs [123,129]. pS910 and pS935 residues play an important role in modulating 14-3-3 chaperone binding [130]. Loss of this binding results in alterations in protein localisation and stability, whereas the role of pS955 and pS973 is currently unknown [129,131]. Initial studies have shown that pS910 and pS935 levels are not statistically different in PD, as measured by immunoblotting, suggesting an unlikely direct contribution of these modifications to PD pathology [123]. On the other hand, the application of specific LRRK2 kinase inhibitors *ex vivo*, such as PF06447475, has proven to be promising for drug treatment evaluations as they cause a dose-dependent loss of phosphorylation at these sites [129]. In this regard, pS910 and pS935 may not serve as reliable biomarker targets *per se* (i.e., as markers of disease progression); however, can be utilised in testing compound targeting in therapeutics. The inability to detect changes in total or phosphorylated LRRK2 levels in PBMCs raises questions as to whether there is a masking effect due to the heterogeneity of these cells; thus, the different subcellular types should be purified and examined separately for clarification in future experiments [122–124].

Upstream effectors and the pathways implicated in phosphorylating/dephosphorylating LRRK2 are largely unknown, with some likely candidates mentioned elsewhere [132]. While additional studies are needed, it has been suggested that the endogenous downstream LRRK2 substrates, Rab GTPases strongly involved in vesicular trafficking, can be used as a direct measure of kinase activity [133–135]. Phosphoproteomic screening identified numerous Rabs as substrates of LRRK2, among which Rab10 has attracted the greatest attention for biomarker studies [135]. An initial report showing that these proteins are expressed in PBMCs came from a small cohort study of 10 healthy donors [126]. Phosphorylated Rab10 (pRab10) was selectively attenuated upon treatment with Lu AF58786 and PFE 360 LRRK2 kinase inhibitors, indicating that this phosphorylation is LRRK2 dependent [126]. Levels of pRab12 were also examined; however, dephosphorylation did not correlate with LRRK2 inhibition [126]. Consistent results with previous findings for reduced pS935 levels were also shown, therefore, it was demonstrated that pRab10 quantification can potentially be used along with pS910 and pS935 in target engagement assays [126]. No significant pRab10 differences have been observed in PBMCs and neutrophils of PD patients and controls, implying that pRab10 may not be suitable as a PD biomarker *per se* in these cells, however, more work is needed with larger multi-centre cohorts [122,127]. MLI-2 and PF-06447475 inhibitors reduce pRab10 and pS935 in both PD and controls to a similar extent, whereas total levels remain unaffected, highlighting once again their potential use as peripheral drug targets [122,127]. Higher total LRRK2 levels have been observed in PD neutrophils versus controls, whereas this difference was not observed in PBMCs [127]. Of note, one disadvantage of using neutrophils is that purification may be contaminated with CD16⁺ monocytes, possibly contributing to discrepancies in interpretation of the findings [136].

Unexpectedly, pS935 levels were reduced in G2019S PD versus iPD patients, and when normalised to total LRRK2 this reduction was also apparent between G2019S PD individuals versus G2019S healthy carriers and healthy controls [125]. We know from previous studies in overexpression systems of this mutant that it does not typically alter phosphorylation at this site [123]. These results demonstrate that this specific mutation negatively regulates pS935 by likely unique mechanisms in these cells, and that the high levels of over-expression in experimental models may obscure small reductions in pS935-LRRK2 levels in G2019S-expressing cells. There was no indication of correlation between motor symptoms or cognitive decline and low pS935 levels [125]. This was one of a few experiments where ELISA was used instead of immunoblotting, suggesting that more sensitive assays can reveal changes that otherwise may be masked. Similar experiments should be carried out in larger cohorts to assess pS935 levels in relation to pRab10 readouts for comparison. Similar studies

should also be conducted in carriers of other pathogenic mutations in LRRK2, in order to determine if similar trends are observed as for G2019S-LRRK2.

The most reliable way of measuring LRRK2 kinase activity directly is by quantifying the levels of auto-phosphorylation at pS1292 residue, as described in many experimental models (e.g., [114]). This is robustly detected in CSF and urinary exosomes, as described in the subsections below [117,119,128]. Peculiarly, no group has reported this modification in LRRK2 from PBMCs, and it is unknown whether phosphorylation at this site is detectable in LRRK2 purified from monocytes or neutrophils. This may mean that levels are below the detection limit of the assays currently available, or that this residue is not phosphorylated in these cells.

4.3. CSF

LRRK2 quantification with respect to CSF is mainly based on exosomal isolation rather than pure CSF itself. Isolation is achieved by various methods, as described elsewhere [137,138]. As mentioned previously in the AS section, monitoring changes in protein expression levels and activity in CSF originating exosomes can be used as a tool for PD biomarker studies. LRRK2-positive exosomes can emerge from various cell types in a mechanism that is 14-3-3 chaperone-dependent [118,139]. Levels of pS1292 have been detected in G2019S related PD and healthy groups, although no significant changes were observed between them [128]. The signal of pS1292 was highly saturated and any differences could have been underestimated [128]. By comparing these levels with corresponding pS1292 in urine exosomes (described in next the section) from the same participants, the signal was much more robust in CSF (~10-fold), whereas no correlation was found for total LRRK2 levels between the two biofluids [128]. What this possibly tells us about LRRK2 is that its activity is higher in the brain than in the periphery.

4.4. Urine

Urine is much more accessible and can be obtained at larger quantities than any other biofluid. Although the majority of exosomes in urine were thought to originate from the kidney, it is now evident from mass spectrometry and bioinformatics that there is a great diversity of the proteins present, coming from many tissues around the body, and PD-related proteins scored the highest according to PANTHER analysis [139]. However, as shown by cryo-EM, during the isolation process larger vesicles (>200 nm) can also be recovered, resulting in a heterogeneous population, potentially introducing an unknown variable [139]. Even so, LRRK2 is present in the lumen of urinary exosomes at detectable levels [98]. Intriguing findings came from a study where PD risk could be predicted by measuring pS1292 levels in a small cohort of male G2019S carriers with PD, which were ~5-fold higher compared to PD non-carriers when normalised to total LRRK2 [117]. Replication of these results in a larger cohort of male carriers with PD versus carriers without PD was successful, with ~3.6-fold elevation [118]. Most importantly, the same outcome was reproduced in urinary exosomes coming from iPD male and female patients versus healthy participants, with a ~1.5-fold difference [119]. Sex differences were also observed, where males had augmented pS1292 levels compared to females and were generally in correlation with poor clinical test scores [119]. This finding emphasizes once more the role of LRRK2 and its pS1292 modification as a potential therapeutic biomarker target in both iPD and G2019S-related PD cases. Furthermore, a relationship between auto-phosphorylation levels and medication was clear [119]. In another experiment, the contribution of sex was apparent, with total LRRK2 levels being higher in females, irrespective of disease status, but still without any significant differences between PD and healthy groups [117,119,120]. In PBMCs, however, there is an apparent reversal of the sex differences, with higher LRRK2 expression levels detected in males [14].

Lastly, evidence of Rab GTPase presence or phosphorylation in CSF or urinary exosomes has not yet been reported. A unique relationship between one specific Rab GTPase was recently described for Rab29, which is not only a substrate of LRRK2 but also an upstream activator. A proposed model demonstrated that both interact in a loop where Rab29 activates LRRK2, which then, in turn, phosphorylates Rab29 [140–142]. Whether Rab29 exist in human biofluids remains to be elucidated, and if it does, important findings may be disclosed due to its binary role.

5. Therapies Targeting GCase

Since their identification [23,143,144], heterozygous mutations in the gene encoding β -glucocerebrosidase (GCase), *GBA1* (GBA), are considered the greatest genetic risk factor for developing PD [9,145] and it is estimated that between 5%–25% of all PD patients carry GBA mutations [146]. Furthermore, PD patients that do not carry a GBA mutation demonstrate reduced GCase activity in the striatum and substantia nigra in conjunction with an accumulation of AS and alterations in lysosomal autophagy and lipid metabolism [147]. Phenotypically, iPD and GBA-PD are almost indistinguishable, except for an accelerated progression of motor and non-motor symptoms in GBA-PD patients [148,149]. GCase deficiency can lead to the accumulation of AS via the stabilization of oligomers, which in turn can cause a further decrease in GCase activity, leading to a bidirectional positive feedback loop effect [150]. This “toxic” relationship has been further advanced in vitro and in vivo and the prevailing hypothesis posits that GCase insufficiency leads to lysosomal dysfunction and subsequent proteinopathy in synucleinopathies [151]. Fascinatingly, GCase activity was found to be altered in LRRK2 patient blood samples [152], suggesting, along with the abovementioned data, an intriguing convergence of AS, LRRK2 and GBA.

GCase hydrolyzes glucosylceramide into ceramide and glucose. Homozygous or compound heterozygous *GBA1* mutations causes Gaucher disease (GD), a lysosomal storage disorder that leads to glycolipid accumulation in lysosomes. Although effective therapies are available for GD, including enzyme replacement therapy and substrate reduction therapy (i.e., glucosylceramide synthase inhibitors), they do not demonstrate target engagement in the CNS. Similar strategies currently in development for GBA-PD include boosting GCase activity via gene therapy, GCase small-molecule chaperones (SMCs) or glucosylceramide synthase inhibitors [153] (summarized in Table 3).

Gene therapy targeting the *GBA1* gene using an AAV-9 vector (PR001A) for use in GBA-PD is currently in Phase I, the PROPEL study, being tested for the safety of intra-cisternal administration in PD patients with at least one pathogenic *GBA1* mutation (Prevail Therapeutics; NCT04127578), while there are currently three ongoing phase II studies targeting GCase. The EMA-approved mucolytic, ambroxol, is an inhibitory SMC, i.e., it binds to the active site of GCase, inhibiting its activity, and it has demonstrated increased GCase activity in vivo along with a reduction in AS levels and target engagement in patients [154,155]. It is now being tested for repurposing in PD in the AiM-PD trial (University College London) for safety, tolerability and pharmacodynamic effects (NCT012941822) and in a separate trial for PD dementia (NCT02914366). LTI-291 is a non-inhibitory SMC (i.e., binds to a different site, not affecting activity) that is currently being tested in GBA-PD patients (Lysosomal Therapeutics; NTR7299). Venglustat (Ibiglustat, GZ667161/SAR402671) is a glucosylceramide synthase inhibitor, thus inhibiting the synthesis of glycosphingolipids. Venglustat is currently being assessed in the double-blind, placebo-controlled MOVES-PD study for efficacy and safety in patients with early-stage PD carrying a *GBA1* mutation (NCT02906020).

GBA-based therapeutic targets simultaneously underline the importance of patient stratification and sub-population specific clinical trials and the need for biomarker assays (i.e., GCase activity) for the assessment of target engagement and efficacy. If and when these trials demonstrate sufficient target engagement and efficacy, evidence suggests that such treatments may hold potential for use more broadly in idiopathic forms of PD.

Table 3. Glucocerebrosidase (GBA) therapeutics.

Compound	Mechanism of Action	Clinical Indication	Patient Group	Sponsors	Clinical Phase	Time Period (Ongoing)	Clinical Trial
PR001A	GBA1: PD-GBA AAV-9 gene therapy	GBA-PD	GBA-PD	Prevail therapeutics	1/2 [^] : safety, tolerability, immunogenicity, biomarkers, efficacy	10/2019	PROPEL NCT04127578
Ambroxol *	Glucocerebrosidase chaperone: inhibitory SMC for GCase	Disease-modify PD PD dementia	PD PD + dementia	University College, London	2: Safety, tolerability, PD 2 Safety, tolerability, efficacy	12/2016–04/2018 (in press) 11/2015-	AiM-PD NCT02941822 NCT02914366
LTI-291	Non-inhibitory SMC	GBA-PD	GBA-PD	Lysosomal Therapeutics	2: Safety, tolerability, PK/PD	06/2018	NTR7299 (Netherlands Trial Register)
Venglustat (Ibiclustat; GZ667161/S AR402671)	Glucosylceramide synthase inhibitor	GBA-PD	GBA-PD	Sanofi Genzyme	2: Safety, tolerability, efficacy	12/2016	MOVES-PD NCT02906020

Table 3 Glucocerebrosidase (GBA)-based disease-modifying treatment strategies and their current progress in clinical trials. * Repurposed drugs, ^ first in-human study administering PR001A (gene therapy) in GBA-PD patients with a sham procedure as control; PK = pharmacokinetics, PD = pharmacodynamics; Clinical phases: 1 =Phase 1, clinical trials that focus on drug safety typically conducted with healthy volunteers to determine the drug's pharmacokinetics and most frequent and serious adverse events; Phase 2, clinical trials that focus on the drug's effectiveness in patients populations while continuing to study its safety and short-term adverse events.

5.1. GCase-Targeted Biomarkers

In most biomarker experiments that have been carried out in blood or CSF biofluids, GCase activity is generally lower in PD, irrespective of mutations. Based on the available findings, there is an indicative link between GCase, AS and LRRK2 PD pathology, as will be discussed here, and defining how the different pathways converge and interact in the disease is crucial for therapeutics. Hence, GCase activity has also been looked at in patients carrying mutations in AS or LRRK2 (see Summary Box 3).

Box 3: Overview of GCase-targeted biomarkers.

Blood

- GBA PD patients have lower GCase activity compared to non-carriers and healthy controls in dried blood spots [152].
- PD patients without the GBA mutation show a moderate decrease of GCase enzymatic activity [152] while lower activity has also been observed in polymorphic GBA variants though without significant change between iPD patients and healthy subjects [156].
- G2019S LRRK2 PD patients display higher GCase enzymatic activity [152].
- GBA mutation and polymorphic carriers have increased oligomeric AS species accumulated in plasma compared to iPD and healthy controls [157].
- There is defective GCase activity in the PBMCs of GBA patients versus A53T SNCA carriers or patients with iPD [156].
- GCase activity is diminished in monocytes of iPD patients [157].
- The higher levels of AS accumulation reportedly found in plasma exosomes of iPD patients versus healthy subjects are inversely proportional to GCase activity when normalised to free AS in plasma, with total AS levels in plasma remaining unaffected [158].

CSF

- Lower GCase activity, reported in GBA PD carriers and non-carriers versus healthy controls [159], is inversely related with higher total and oligomeric AS [160].

5.2. Blood

GCCase activity was lower in GBA-PD patients versus non-carriers and healthy controls in dried blood spots and non-carriers alone were also characterized by a moderate decrease [152]. However, this group suggested that although it can contribute to pathogenesis, lower enzymatic activity is insufficient to cause PD; hence, it is not a reliable biomarker per se but can be considered as a tool to assess therapeutic target engagement [152]. In contrast, G2019S LRRK2 PD participants showed higher enzymatic activity compared with non-carriers and healthy controls, indicating that autophago-lysosomal pathways are differently regulated in this phenotype [152]. Lower activity has also been observed to a lesser extent in polymorphic *GBA1* variants; however, no significant change regarding iPD and healthy subjects was noted in this study [137]. Defective enzymatic activity was also present in PBMCs of GBA patients but not in A53T *SNCA* carriers or iPD [156]. Nevertheless, activity was diminished in the monocytes of iPD patients, a PBMC sub-population that expresses very high levels of GCCase [127,158]. This difference can be attributed to a masking effect due to the heterogeneity of PBMCs.

As GBA mutations lead to loss of enzymatic function and autophago-lysosomal defects, it is speculated that AS cannot be sequestered for degradation effectively, building up in PD patient brains and this build up may further inhibit GCCase activity [145,158]. This could explain the increased oligomeric species accumulated in plasma of *GBA* mutation and polymorphic carriers versus iPD and healthy controls as an attempt towards cell clearance [157]. Higher levels of AS accumulation have also been measured in plasma exosomes of iPD patients versus healthy subjects and this was inversely proportional to GCCase activity when normalised to free AS in plasma [159]. On the other hand, total AS levels in plasma were unaffected [159]. This finding further highlights that enzymatic activity insufficiency is not only present in GBA PD but also in iPD. Additionally, studying protein levels in exosomes might be a more appropriate way to designate differences between health and disease rather than in free biofluids in general, where a variety of substances circulate, potentially masking any changes.

5.3. CSF

Fewer studies have been conducted in isolated CSF. Like in blood, there is lower GCCase activity in CSF isolated from both GBA PD carriers and non-carriers when compared to healthy controls [160]. Furthermore, there is an established inverse relationship between GCCase activity and AS levels, with lower activity, particularly early in the disease, and higher total and oligomeric AS [161]. As mentioned above, amroxolol is a promising repurposed compound that recently entered phase II clinical trials [155]. CSF from GBA PD patients demonstrated increased GCCase levels and activity and an increase in AS concentration [155]. This may again imply an efflux-clearing mechanism of AS from the brain to the periphery, similar to that suggested in the blood [155].

6. Outstanding Issues and Questions

Idiopathic PD: Is there Value in Targeting Genetic PD Players?

In addition to the loss of dopamine-producing neurons of the substantia nigra pars compacta, a key pathological hallmark of PD is the appearance of AS-positive cytoplasmic proteinaceous inclusions known as LBs. These structures have been detected in surviving DA neurons, and in most forms (idiopathic as well as familial) of PD. Notable exceptions to this include cases of familial PD associated with mutations in the *LRRK2* gene, where the neuropathology is heterogeneous [162]; although some pathology reports have suggested that *LRRK2* can also be sequestered within LBs [163]. Thus, even in the absence of pathogenic mutations or multiplications in the *SNCA* gene, deposition of AS in surviving neurons is a common pathological finding in PD. While it is still unclear whether these dense proteinaceous structures are toxic, or simply discrete, confined, aggregates; the implication remains that AS plays a role in general, in PD. It has been postulated that the toxic conformation of AS is not the aggregated species detected in LBs, but rather soluble fibrils of the

protein, which can also be transmitted trans-cellularly, possibly facilitating the progression of the disease. Thus, as in cases of familial PD attributed to mutations in the *SNCA* gene, therapeutic strategies targeting AS may be of benefit in iPD cases where the pathological involvement of AS also plays a role in disease pathogenesis or progression.

In addition to AS, another common autosomal dominant familial form of PD, as discussed above, is that associated with mutations in the *LRRK2* gene, located on chromosome 12 [99,100]. There are several lines of evidence implicating changes in *LRRK2* activity in iPD as well. First, in 2016, the group of Andrew West published two studies [117,119] assessing changes in *LRRK2* auto-phosphorylation (at Ser1292), as a proxy index of kinase activity, in urinary exosomes/extra-cellular vesicles (EVs). In the second of these two studies, the group reported that pS1292-*LRRK2* levels were elevated in iPD, compared to healthy controls [119]. This was the first indication that *LRRK2* activity is altered in forms of PD not associated with pathogenic mutations, which are known to increase kinase activity. Secondly, in an assessment of post-mortem tissue from iPD brain, Di Maio and colleagues applied the proximity ligation assay to demonstrate enhanced auto-phosphorylation of *LRRK2* itself (S1292) and phosphorylation of its major substrate Rab10 (T73) as indices of *LRRK2* kinase activation specifically in dopamine neurons in the ventral midbrain [164]. In addition, their findings of decreased pS129 AS levels and *LRRK2* kinase activity in rotenone-administered rats, following *LRRK2* kinase inhibition, also demonstrates the interplay between *LRRK2* and AS and supports the potential use of *LRRK2* inhibitors in iPD. Finally, modulation of GCase activity is inversely correlated with AS levels in both GBA-PD and iPD and *LRRK2* mutations also affect GCase levels, further demonstrating an interaction amongst the three gene targets. The reported milder phenotype of double *LRRK2* and *GBA1* variant carriers vs. GBA-PD further complicates this interrelationship but at the same time, provides additional evidence for bidirectional modulation and potential for broader use of specifically targeted therapeutics.

Overall, research centered on the underlying genetics, gene target convergence and their potential role in iPD should remain a top priority. Our growing knowledge of PD genetic architecture and the consensus that initially targeting specific subpopulations will lead to greater clinical success, has also created hope for a future of “precision neurology” for patients [165]. Even though we still have a lot to learn, armored with this constantly expanding knowledge base and innovative technologies, disease modification constitutes a more tangible goal than ever before. However, one of the many challenges hindering clinical trial success is the lack of biomarkers that can aid decision-making. Aside from the importance of environmental factors in PD, a new conceptual model consisting of triggers (agents that can initiate the disease process), facilitators (factors that aid disease spread in the CNS) and aggravators (elements that exacerbate pathology and spread disease beyond the basal ganglia), emphasizes the temporal importance of disease events [166] and therapeutic targeting (i.e., applying therapeutics when the target is most active); again, making evident the importance and urgency of biomarker development. Inevitably, a multifaceted approach to early/timely diagnosis, prognosis and treatment is required in complex, multifactorial and slowly progressing diseases such as PD and other neurodegenerative disorders. Perhaps we can learn a few lessons from multimodal models of diagnosis and treatment that have recently been proposed for vascular-related and mixed (vascular + degenerative) cognitive disorders [167,168] based on both pharmacological and non-pharmacological strategies. Adapted for PD, this first approach would include combinatorial drug therapy (with both existing and new disease-modifying drugs) targeting neuronal death and axonal loss, brain plasticity and compensatory mechanisms and protein misfolding in addition to non-pharmacological strategies such as transcranial brain stimulation and physical activity [167]. Similar to what we have proposed for PD, the second model highlights the usefulness of a panel of markers, including biofluids but also histopathological, imaging, and neurophysiological findings to enable prediction of “at risk” brains [168]. Altogether, translational medicine in PD research will benefit from clinical protocols based on multiple animal models (i.e., to maximally mimic the complete spectrum of disease) to improve patient stratification and the use of biomarkers in these models and in the clinic as outcome measures for disease-modifying therapeutic development and refinement.

7. Conclusions

As PD is a complex and multifactorial disease, the future research agenda must include a multidimensional approach involving individualized patient profiling and consideration of additional factors including sex and comorbidities. In this focused review, we have covered aspects of disease-modifying therapies relevant primarily to PD linked genetically to *SNCA*, *LRRK2*, and *GBA1* variants. While PD is a profoundly more complex disease than implied by this focus, there is ample evidence that each of the protein products of these genes may also play a role in multiple aspects of disease pathogenesis and/or progression, including mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, impaired autophagy, axonal transport dysfunction, synaptic alterations and neuroinflammation, also affected in the more common idiopathic form of the disease. Further, it is apparent that a convergence exists between these proteins at the level of the lysosomal degradation system (e.g., [169]), providing further targets for novel therapeutic candidates as well as biomarker development—a critical component of drug discovery efforts. Along these lines, we have tried to emphasize, for each of the three targets, the need for validated, quantitative biomarkers that can track or predict disease progression, or serve as markers of target engagement in clinical trials of new therapies.

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References

1. Dorsey, E.R.; Bloem, B.R. The Parkinson Pandemic-A Call to Action. *JAMA Neurol.* **2018**, *75*, 9–10, doi:10.1001/jamaneurol.2017.3299.
2. Postuma, R.B.; Aarsland, D.; Barone, P.; Burn, D.J.; Hawkes, C.H.; Oertel, W.; Ziemssen, T. Identifying prodromal Parkinson's disease: Pre-motor disorders in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2012**, *27*, 617–626, doi:10.1002/mds.24996.
3. Gasser, T. Mendelian forms of Parkinson's disease. *Biochim. Biophys. Acta* **2009**, *1792*, 587–596, doi:10.1016/j.bbdis.2008.12.007.
4. Blauwendraat, C.; Heilbron, K.; Vallerga, C.L.; Bandres-Ciga, S.; von Coelln, R.; Pihlstrom, L.; Simon-Sanchez, J.; Schulte, C.; Sharma, M.; Krohn, L.; et al. Parkinson's disease age at onset genome-wide association study: Defining heritability, genetic loci, and alpha-synuclein mechanisms. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2019**, *34*, 866–875, doi:10.1002/mds.27659.
5. Cali, F.; Cantone, M.; Cosentino, F.I.I.; Lanza, G.; Ruggeri, G.; Chiavetta, V.; Salluzzo, R.; Ragalmuto, A.; Vinci, M.; Ferri, R. Interpreting Genetic Variants: Hints from a Family Cluster of Parkinson's Disease. *J. Parkinson's Dis.* **2019**, *9*, 203–206, doi:10.3233/JPD-171292.
6. Polymeropoulos, M.H.; Lavedan, C.; Leroy, E.; Ide, S.E.; Dehejia, A.; Dutra, A.; Pike, B.; Root, H.; Rubenstein, J.; Boyer, R.; et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **1997**, *276*, 2045–2047, doi:10.1126/science.276.5321.2045.
7. Li, J.Q.; Tan, L.; Yu, J.T. The role of the LRRK2 gene in Parkinsonism. *Mol. Neurodegener.* **2014**, *9*, 47, doi:10.1186/1750-1326-9-47.
8. Sidransky, E. Gaucher disease and parkinsonism. *Mol. Genet. Metab.* **2005**, *84*, 302–304, doi:10.1016/j.ymgme.2004.11.007.
9. Sidransky, E.; Lopez, G. The link between the GBA gene and parkinsonism. *Lancet Neurol.* **2012**, *11*, 986–998, doi:10.1016/S1474-4422(12)70190-4.

10. Meoni, S.; Macerollo, A.; Moro, E. Sex differences in movement disorders. *Nat. Rev. Neurol.* **2020**, *16*, 84–96, doi:10.1038/s41582-019-0294-x.
11. Caranci, G.; Piscopo, P.; Rivabene, R.; Traficante, A.; Riozzi, B.; Castellano, A.E.; Ruggieri, S.; Vanacore, N.; Confaloni, A. Gender differences in Parkinson's disease: Focus on plasma alpha-synuclein. *J. Neural Transm. (Vienna, Austria: 1996)* **2013**, *120*, 1209–1215, doi:10.1007/s00702-013-0972-6.
12. Shu, L.; Zhang, Y.; Pan, H.; Xu, Q.; Guo, J.; Tang, B.; Sun, Q. Clinical Heterogeneity Among LRRK2 Variants in Parkinson's Disease: A Meta-Analysis. *Front. Aging Neurosci.* **2018**, *10*, 283, doi:10.3389/fnagi.2018.00283.
13. San Luciano, M.; Wang, C.; Ortega, R.A.; Giladi, N.; Marder, K.; Bressman, S.; Saunders-Pullman, R.; Michael, J.F.F.L.C. Sex differences in LRRK2 G2019S and idiopathic Parkinson's Disease. *Ann. Clin. Transl. Neurol.* **2017**, *4*, 801–810, doi:10.1002/acn3.489.
14. Melachroinou, K.; Kang, M.; Liong, C.; Narayan, S.; Levers, N.; Joshi, N.; Kopil, K.; Hutten, S.J.; Baptista, M.A.; Padmanabhan, S.; et al. Elevated in vitro kinase activity in PBMCs of LRRK2 G2019S carriers: A novel ELISA-based method. *Mov. Disord.: Off. J. Mov. Disord. Soc.* **2020**, in press.
15. Swan, M.; Doan, N.; Ortega, R.A.; Barrett, M.; Nichols, W.; Ozelius, L.; Soto-Valencia, J.; Boschung, S.; Deik, A.; Sarva, H.; et al. Neuropsychiatric characteristics of GBA-associated Parkinson disease. *J. Neurol. Sci.* **2016**, *370*, 63–69, doi:10.1016/j.jns.2016.08.059.
16. Pagano, G.; Niccolini, F.; Politis, M. Imaging in Parkinson's disease. *Clin. Med.* **2016**, *16*, 371–375, doi:10.7861/clinmedicine.16-4-371.
17. He, R.; Yan, X.; Guo, J.; Xu, Q.; Tang, B.; Sun, Q. Recent Advances in Biomarkers for Parkinson's Disease. *Front. Aging Neurosci.* **2018**, *10*, 305, doi:10.3389/fnagi.2018.00305.
18. Tang, Y.; Meng, L.; Wan, C.M.; Liu, Z.H.; Liao, W.H.; Yan, X.X.; Wang, X.Y.; Tang, B.S.; Guo, J.F. Identifying the presence of Parkinson's disease using low-frequency fluctuations in BOLD signals. *Neurosci. Lett.* **2017**, *645*, 1–6, doi:10.1016/j.neulet.2017.02.056.
19. Akdemir, U.O.; Tokcaer, A.B.; Karakus, A.; Kapucu, L.O. Brain 18F-FDG PET imaging in the differential diagnosis of parkinsonism. *Clin. Nucl. Med.* **2014**, *39*, e220–e226, doi:10.1097/RLU.0000000000000315.
20. Ni, Z.; Bahl, N.; Gunraj, C.A.; Mazzella, F.; Chen, R. Increased motor cortical facilitation and decreased inhibition in Parkinson disease. *Neurology* **2013**, *80*, 1746–1753, doi:10.1212/WNL.0b013e3182919029.
21. Jackson, N.; Cole, S.R.; Voytek, B.; Swann, N.C. Characteristics of Waveform Shape in Parkinson's Disease Detected with Scalp Electroencephalography. *eNeuro* **2019**, *6*, doi:10.1523/ENEURO.0151-19.2019.
22. Singleton, A.B.; Farrer, M.; Johnson, J.; Singleton, A.; Hague, S.; Kachergus, J.; Hulihan, M.; Peuralinna, T.; Dutra, A.; Nussbaum, R.; et al. alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **2003**, *302*, 841, doi:10.1126/science.1090278.
23. Spillantini, M.G.; Schmidt, M.L.; Lee, V.M.; Trojanowski, J.Q.; Jakes, R.; Goedert, M. Alpha-synuclein in Lewy bodies. *Nature* **1997**, *388*, 839–840, doi:10.1038/42166.
24. Brundin, P.; Melki, R. Prying into the Prion Hypothesis for Parkinson's Disease. *J. Neurosci.* **2017**, *37*, 9808–9818, doi:10.1523/JNEUROSCI.1788-16.2017.
25. Desplats, P.; Lee, H.J.; Bae, E.J.; Patrick, C.; Rockenstein, E.; Crews, L.; Spencer, B.; Masliah, E.; Lee, S.J. Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 13010–13015, doi:10.1073/pnas.0903691106.
26. Li, J.Y.; Englund, E.; Holton, J.L.; Soulet, D.; Hagell, P.; Lees, A.J.; Lashley, T.; Quinn, N.P.; Rehncrona, S.; Bjorklund, A.; et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat. Med.* **2008**, *14*, 501–503, doi:10.1038/nm1746.
27. Luk, K.C.; Kehm, V.; Carroll, J.; Zhang, B.; O'Brien, P.; Trojanowski, J.Q.; Lee, V.M. Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science* **2012**, *338*, 949–953, doi:10.1126/science.1227157.
28. Koprach, J.B.; Kalia, L.V.; Brotchie, J.M. Animal models of alpha-synucleinopathy for Parkinson disease drug development. *Nat. Rev. Neurosci.* **2017**, *18*, 515–529, doi:10.1038/nrn.2017.75.
29. Peelaerts, W.; Bousset, L.; Van der Perren, A.; Moskalyuk, A.; Pulizzi, R.; Giugliano, M.; Van den Haute, C.; Melki, R.; Baekelandt, V. alpha-Synuclein strains cause distinct synucleinopathies after local and systemic administration. *Nature* **2015**, *522*, 340–344, doi:10.1038/nature14547.
30. Cronin, K.D.; Ge, D.; Manning, P.; Linnertz, C.; Rossoshek, A.; Orrison, B.M.; Bernard, D.J.; El-Agnaf, O.M.; Schlossmacher, M.G.; Nussbaum, R.L.; et al. Expansion of the Parkinson disease-associated SNCA-Rep1 allele upregulates human alpha-synuclein in transgenic mouse brain. *Hum. Mol. Genet.* **2009**, *18*, 3274–3285, doi:10.1093/hmg/ddp265.

31. Fuchs, J.; Tichopad, A.; Golub, Y.; Munz, M.; Schweitzer, K.J.; Wolf, B.; Berg, D.; Mueller, J.C.; Gasser, T. Genetic variability in the SNCA gene influences alpha-synuclein levels in the blood and brain. *FASEB J.: Off. Publ. Fed. Am. Soc. Exp. Biol.* **2008**, *22*, 1327–1334, doi:10.1096/fj.07-9348com.
32. Mata, I.F.; Shi, M.; Agarwal, P.; Chung, K.A.; Edwards, K.L.; Factor, S.A.; Galasko, D.R.; Ginghina, C.; Griffith, A.; Higgins, D.S.; et al. SNCA variant associated with Parkinson disease and plasma alpha-synuclein level. *Arch. Neurol.* **2010**, *67*, 1350–1356, doi:10.1001/archneurol.2010.279.
33. Simon-Sanchez, J.; Schulte, C.; Bras, J.M.; Sharma, M.; Gibbs, J.R.; Berg, D.; Paisan-Ruiz, C.; Lichtner, P.; Scholz, S.W.; Hernandez, D.G.; et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat. Genet.* **2009**, *41*, 1308–1312, doi:10.1038/ng.487.
34. Soldner, F.; Stelzer, Y.; Shivalila, C.S.; Abraham, B.J.; Latourelle, J.C.; Barrasa, M.I.; Goldmann, J.; Myers, R.H.; Young, R.A.; Jaenisch, R. Parkinson-associated risk variant in distal enhancer of alpha-synuclein modulates target gene expression. *Nature* **2016**, *533*, 95–99, doi:10.1038/nature17939.
35. Mercuri, E.; Darras, B.T.; Chiriboga, C.A.; Day, J.W.; Campbell, C.; Connolly, A.M.; Iannaccone, S.T.; Kirschner, J.; Kuntz, N.L.; Saito, K.; et al. Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* **2018**, *378*, 625–635, doi:10.1056/NEJMoa1710504.
36. Wild, E.J.; Tabrizi, S.J. Therapies targeting DNA and RNA in Huntington's disease. *Lancet Neurol.* **2017**, *16*, 837–847, doi:10.1016/S1474-4422(17)30280-6.
37. Cole, T.A.; Zhao, H.; Collier, T.J.; Sandoval, I.; Sortwell, C.E.; Steece-Collier, K.; Daley, B.F.; Booms, A.; Lipton, J.; Welch, M.; et al. Alpha-synuclein antisense oligonucleotides as a disease-modifying therapy for Parkinson's disease. *BioRxiv* **2019**, doi:10.1101/830554.
38. Mittal, S.; Bjornevik, K.; Im, D.S.; Flierl, A.; Dong, X.; Locascio, J.J.; Abo, K.M.; Long, E.; Jin, M.; Xu, B.; et al. beta2-Adrenoreceptor is a regulator of the alpha-synuclein gene driving risk of Parkinson's disease. *Science* **2017**, *357*, 891–898, doi:10.1126/science.aaf3934.
39. Hopfner, F.; Wod, M.; Hoglinger, G.U.; Blaabjerg, M.; Rosler, T.W.; Kuhlenbaumer, G.; Christensen, K.; Deuschl, G.; Pottegard, A. Use of beta2-adrenoreceptor agonist and antagonist drugs and risk of Parkinson disease. *Neurology* **2019**, *93*, e135–e142, doi:10.1212/WNL.0000000000007694.
40. Searles Nielsen, S.; Gross, A.; Camacho-Soto, A.; Willis, A.W.; Racette, B.A. beta2-adrenoreceptor medications and risk of Parkinson disease. *Ann. Neurol.* **2018**, *84*, 683–693, doi:10.1002/ana.25341.
41. Charvin, D.; Di Paolo, T.; Bezard, E.; Gregoire, L.; Takano, A.; Duvey, G.; Pioli, E.; Halldin, C.; Medori, R.; Conquet, F. An mGlu4-Positive Allosteric Modulator Alleviates Parkinsonism in Primates. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2018**, *33*, 1619–1631, doi:10.1002/mds.27462.
42. Vekrellis, K.; Xilouri, M.; Emmanouilidou, E.; Stefanis, L. Inducible over-expression of wild type alpha-synuclein in human neuronal cells leads to caspase-dependent non-apoptotic death. *J. Neurochem.* **2009**, *109*, 1348–1362, doi:10.1111/j.1471-4159.2009.06054.x.
43. Price, D.L.; Koike, M.A.; Khan, A.; Wrasidlo, W.; Rockenstein, E.; Masliah, E.; Bonhaus, D. The small molecule alpha-synuclein misfolding inhibitor, NPT200-11, produces multiple benefits in an animal model of Parkinson's disease. *Sci. Rep.* **2018**, *8*, 16165, doi:10.1038/s41598-018-34490-9.
44. Hebron, M.L.; Lonskaya, I.; Moussa, C.E. Nilotinib reverses loss of dopamine neurons and improves motor behavior via autophagic degradation of alpha-synuclein in Parkinson's disease models. *Hum. Mol. Genet.* **2013**, *22*, 3315–3328, doi:10.1093/hmg/ddt192.
45. Pagan, F.; Hebron, M.; Valadez, E.H.; Torres-Yaghi, Y.; Huang, X.; Mills, R.R.; Wilmarth, B.M.; Howard, H.; Dunn, C.; Carlson, A.; et al. Nilotinib Effects in Parkinson's disease and Dementia with Lewy bodies. *J. Parkinson's Dis.* **2016**, *6*, 503–517, doi:10.3233/JPD-160867.
46. Simuni, T.; Fiske, B.; Merchant, K.; Coffey, C.; Klingner, E.; Caspell-Garcia, C.; Lafontant, D.E.; Matthews, H.; Wyse, R.K.; Brundin, P.; et al. Nilotinib in patients with advanced Parkinson's disease: A randomized phase 2A study (NILO-PD). *medRxiv* **2020**, doi:10.1101/2020.05.11.20093146.
47. El-Agnaf, O.M.; Salem, S.A.; Paleologou, K.E.; Cooper, L.J.; Fullwood, N.J.; Gibson, M.J.; Curran, M.D.; Court, J.A.; Mann, D.M.; Ikeda, S.; et al. Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **2003**, *17*, 1945–1947, doi:10.1096/fj.03-0098fje.
48. El-Agnaf, O.M.; Salem, S.A.; Paleologou, K.E.; Curran, M.D.; Gibson, M.J.; Court, J.A.; Schlossmacher, M.G.; Allsop, D. Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **2006**, *20*, 419–425, doi:10.1096/fj.03-1449com.

49. Emmanouilidou, E.; Elenis, D.; Papasilekas, T.; Stranjalis, G.; Gerozissis, K.; Ioannou, P.C.; Vekrellis, K. Assessment of alpha-synuclein secretion in mouse and human brain parenchyma. *PLoS ONE* **2011**, *6*, e22225, doi:10.1371/journal.pone.0022225.
50. Emmanouilidou, E.; Melachroinou, K.; Roumeliotis, T.; Garbis, S.D.; Ntzouni, M.; Margaritis, L.H.; Stefanis, L.; Vekrellis, K. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J. Neurosci.* **2010**, *30*, 6838–6851, doi:10.1523/JNEUROSCI.5699-09.2010.
51. Brundin, P.; Dave, K.D.; Kordower, J.H. Therapeutic approaches to target alpha-synuclein pathology. *Exp. Neurol.* **2017**, *298*, 225–235, doi:10.1016/j.expneurol.2017.10.003.
52. Jankovic, J.; Goodman, I.; Safirstein, B.; Marmon, T.K.; Schenk, D.B.; Koller, M.; Zago, W.; Ness, D.K.; Griffith, S.G.; Grundman, M.; et al. Safety and Tolerability of Multiple Ascending Doses of PRX002/RG7935, an Anti-alpha-Synuclein Monoclonal Antibody, in Patients with Parkinson Disease: A Randomized Clinical Trial. *JAMA Neurol.* **2018**, *75*, 1206–1214, doi:10.1001/jamaneurol.2018.1487.
53. Brys, M.; Fanning, L.; Hung, S.; Ellenbogen, A.; Penner, N.; Yang, M.; Welch, M.; Koenig, E.; David, E.; Fox, T.; et al. Randomized phase I clinical trial of anti-alpha-synuclein antibody BIIB054. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2019**, *34*, 1154–1163, doi:10.1002/mds.27738.
54. Surmeier, D.J.; Obeso, J.A.; Halliday, G.M. Parkinson's Disease Is Not Simply a Prion Disorder. *J. Neurosci.* **2017**, *37*, 9799–9807, doi:10.1523/JNEUROSCI.1787-16.2017.
55. Hansson, O.; Hall, S.; Ohrfelt, A.; Zetterberg, H.; Blennow, K.; Minthon, L.; Nagga, K.; Londos, E.; Varghese, S.; Majbour, N.K.; et al. Levels of cerebrospinal fluid alpha-synuclein oligomers are increased in Parkinson's disease with dementia and dementia with Lewy bodies compared to Alzheimer's disease. *Alzheimer's Res. Ther.* **2014**, *6*, 25, doi:10.1186/alzrt255.
56. Wang, Y.; Shi, M.; Chung, K.A.; Zabetian, C.P.; Leverenz, J.B.; Berg, D.; Srilijes, K.; Trojanowski, J.Q.; Lee, V.M.; Siderowf, A.D.; et al. Phosphorylated alpha-synuclein in Parkinson's disease. *Sci. Transl. Med.* **2012**, *4*, 121ra120, doi:10.1126/scitranslmed.3002566.
57. Maass, F.; Schulz, I.; Lingor, P.; Mollenhauer, B.; Bahr, M. Cerebrospinal fluid biomarker for Parkinson's disease: An overview. *Mol. Cell. Neurosci.* **2019**, *97*, 60–66, doi:10.1016/j.mcn.2018.12.005.
58. Goldman, J.G.; Andrews, H.; Amara, A.; Naito, A.; Alcalay, R.N.; Shaw, L.M.; Taylor, P.; Xie, T.; Tuite, P.; Henchcliffe, C.; et al. Cerebrospinal fluid, plasma, and saliva in the BioFIND study: Relationships among biomarkers and Parkinson's disease Features. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2018**, *33*, 282–288, doi:10.1002/mds.27232.
59. Sato, H.; Kato, T.; Arawaka, S. The role of Ser129 phosphorylation of alpha-synuclein in neurodegeneration of Parkinson's disease: A review of in vivo models. *Rev. Neurosci.* **2013**, *24*, 115–123, doi:10.1515/revneuro-2012-0071.
60. Mollenhauer, B.; Cullen, V.; Kahn, I.; Krastins, B.; Outeiro, T.F.; Pepivani, I.; Ng, J.; Schulz-Schaeffer, W.; Kretschmar, H.A.; McLean, P.J.; et al. Direct quantification of CSF alpha-synuclein by ELISA and first cross-sectional study in patients with neurodegeneration. *Exp. Neurol.* **2008**, *213*, 315–325, doi:10.1016/j.expneurol.2008.06.004.
61. Tokuda, T.; Salem, S.A.; Allsop, D.; Mizuno, T.; Nakagawa, M.; Qureshi, M.M.; Locascio, J.J.; Schlossmacher, M.G.; El-Agnaf, O.M. Decreased alpha-synuclein in cerebrospinal fluid of aged individuals and subjects with Parkinson's disease. *Biochem. Biophys. Res. Commun.* **2006**, *349*, 162–166, doi:10.1016/j.bbrc.2006.08.024.
62. Majbour, N.K.; Vaikath, N.N.; Eusebi, P.; Chiasserini, D.; Ardah, M.; Varghese, S.; Haque, M.E.; Tokuda, T.; Auinger, P.; Calabresi, P.; et al. Longitudinal changes in CSF alpha-synuclein species reflect Parkinson's disease progression. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2016**, *31*, 1535–1542, doi:10.1002/mds.26754.
63. Ohrfelt, A.; Grognet, P.; Andreasen, N.; Wallin, A.; Vanmechelen, E.; Blennow, K.; Zetterberg, H. Cerebrospinal fluid alpha-synuclein in neurodegenerative disorders—a marker of synapse loss? *Neurosci. Lett.* **2009**, *450*, 332–335, doi:10.1016/j.neulet.2008.11.015.
64. Stewart, T.; Liu, C.; Ghingina, C.; Cain, K.C.; Auinger, P.; Cholerton, B.; Shi, M.; Zhang, J. Cerebrospinal fluid alpha-synuclein predicts cognitive decline in Parkinson disease progression in the DATATOP cohort. *Am. J. Pathol.* **2014**, *184*, 966–975, doi:10.1016/j.ajpath.2013.12.007.
65. Kang, J.H.; Irwin, D.J.; Chen-Plotkin, A.S.; Siderowf, A.; Caspell, C.; Coffey, C.S.; Waligorska, T.; Taylor, P.; Pan, S.; Frasier, M.; et al. Association of cerebrospinal fluid beta-amyloid 1-42, T-tau, P-tau181, and alpha-synuclein levels with clinical features of drug-naïve patients with early Parkinson disease. *JAMA Neurol.* **2013**, *70*, 1277–1287, doi:10.1001/jamaneurol.2013.3861.

66. Lin, C.H.; Yang, S.Y.; Horng, H.E.; Yang, C.C.; Chieh, J.J.; Chen, H.H.; Liu, B.H.; Chiu, M.J. Plasma alpha-synuclein predicts cognitive decline in Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* **2017**, *88*, 818–824, doi:10.1136/jnnp-2016-314857.
67. Duran, R.; Barrero, F.J.; Morales, B.; Luna, J.D.; Ramirez, M.; Vives, F. Plasma alpha-synuclein in patients with Parkinson's disease with and without treatment. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2010**, *25*, 489–493, doi:10.1002/mds.22928.
68. Foulds, P.G.; Diggle, P.; Mitchell, J.D.; Parker, A.; Hasegawa, M.; Masuda-Suzukake, M.; Mann, D.M.; Allsop, D. A longitudinal study on alpha-synuclein in blood plasma as a biomarker for Parkinson's disease. *Sci. Rep.* **2013**, *3*, 2540, doi:10.1038/srep02540.
69. Shi, M.; Liu, C.; Cook, T.J.; Bullock, K.M.; Zhao, Y.; Ginghina, C.; Li, Y.; Aro, P.; Dator, R.; He, C.; et al. Plasma exosomal alpha-synuclein is likely CNS-derived and increased in Parkinson's disease. *Acta Neuropathol.* **2014**, *128*, 639–650, doi:10.1007/s00401-014-1314-y.
70. Vivacqua, G.; Latorre, A.; Suppa, A.; Nardi, M.; Pietracupa, S.; Mancinelli, R.; Fabbrini, G.; Colosimo, C.; Gaudio, E.; Berardelli, A. Abnormal Salivary Total and Oligomeric Alpha-Synuclein in Parkinson's Disease. *PLoS ONE* **2016**, *11*, e0151156, doi:10.1371/journal.pone.0151156.
71. Rani, K.; Mukherjee, R.; Singh, E.; Kumar, S.; Sharma, V.; Vishwakarma, P.; Bharti, P.S.; Nikolajeff, F.; Dinda, A.K.; Goyal, V.; et al. Neuronal exosomes in saliva of Parkinson's disease patients: A pilot study. *Parkinsonism Relat. Disord.* **2019**, *67*, 21–23, doi:10.1016/j.parkreldis.2019.09.008.
72. Cao, Z.; Wu, Y.; Liu, G.; Jiang, Y.; Wang, X.; Wang, Z.; Feng, T. alpha-Synuclein in salivary extracellular vesicles as a potential biomarker of Parkinson's disease. *Neurosci. Lett.* **2019**, *696*, 114–120, doi:10.1016/j.neulet.2018.12.030.
73. Hallett, P.J.; McLean, J.R.; Kartunen, A.; Langston, J.W.; Isacson, O. alpha-Synuclein overexpressing transgenic mice show internal organ pathology and autonomic deficits. *Neurobiol. Dis.* **2012**, *47*, 258–267, doi:10.1016/j.nbd.2012.04.009.
74. Wang, L.; Fleming, S.M.; Chesselet, M.F.; Tache, Y. Abnormal colonic motility in mice overexpressing human wild-type alpha-synuclein. *Neuroreport* **2008**, *19*, 873–876, doi:10.1097/WNR.0b013e3282ffda5e.
75. Ruffmann, C.; Bengoa-Vergniory, N.; Poggiolini, I.; Ritchie, D.; Hu, M.T.; Alegre-Abarrategui, J.; Parkkinen, L. Detection of alpha-synuclein conformational variants from gastro-intestinal biopsy tissue as a potential biomarker for Parkinson's disease. *Neuropathol. Appl. Neurobiol.* **2018**, *44*, 722–736, doi:10.1111/nan.12486.
76. Hong, Z.; Shi, M.; Chung, K.A.; Quinn, J.F.; Peskind, E.R.; Galasko, D.; Jankovic, J.; Zabetian, C.P.; Leverenz, J.B.; Baird, G.; et al. DJ-1 and alpha-synuclein in human cerebrospinal fluid as biomarkers of Parkinson's disease. *Brain* **2010**, *133*, 713–726, doi:10.1093/brain/awq008.
77. Parnetti, L.; Chiasserini, D.; Bellomo, G.; Giannandrea, D.; De Carlo, C.; Qureshi, M.M.; Ardah, M.T.; Varghese, S.; Bonanni, L.; Borroni, B.; et al. Cerebrospinal fluid Tau/alpha-synuclein ratio in Parkinson's disease and degenerative dementias. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2011**, *26*, 1428–1435, doi:10.1002/mds.23670.
78. Brinkmalm, A.; Ohrfelt, A.; Bhattacharjee, P.; Zetterberg, H. Detection of alpha-Synuclein in Biological Samples Using Mass Spectrometry. *Methods Mol. Biol.* **2019**, *1948*, 209–220, doi:10.1007/978-1-4939-9124-2_16.
79. Mollenhauer, B.; Bowman, F.D.; Drake, D.; Duong, J.; Blennow, K.; El-Agnaf, O.; Shaw, L.M.; Masucci, J.; Taylor, P.; Umek, R.M.; et al. Antibody-based methods for the measurement of alpha-synuclein concentration in human cerebrospinal fluid—Method comparison and round robin study. *J. Neurochem.* **2019**, *149*, 126–138, doi:10.1111/jnc.14569.
80. Stewart, T.; Sossi, V.; Aasly, J.O.; Wszolek, Z.K.; Uitti, R.J.; Hasegawa, K.; Yokoyama, T.; Zabetian, C.P.; Leverenz, J.B.; Stoessl, A.J.; et al. Phosphorylated alpha-synuclein in Parkinson's disease: Correlation depends on disease severity. *Acta Neuropathol. Commun.* **2015**, *3*, 7, doi:10.1186/s40478-015-0185-3.
81. Mollenhauer, B.; Trautmann, E.; Taylor, P.; Manninger, P.; Sixel-Doring, F.; Ebentheuer, J.; Trenkwalder, C.; Schlossmacher, M.G. Total CSF alpha-synuclein is lower in de novo Parkinson patients than in healthy subjects. *Neurosci. Lett.* **2013**, *532*, 44–48, doi:10.1016/j.neulet.2012.11.004.
82. Fairfoul, G.; McGuire, L.I.; Pal, S.; Ironside, J.W.; Neumann, J.; Christie, S.; Joachim, C.; Esiri, M.; Evetts, S.G.; Rolinski, M.; et al. Alpha-synuclein RT-QuIC in the CSF of patients with alpha-synucleinopathies. *Ann. Clin. Transl. Neurol.* **2016**, *3*, 812–818, doi:10.1002/acn3.338.

83. Kakuda, K.; Ikenaka, K.; Araki, K.; So, M.; Aguirre, C.; Kajiyama, Y.; Konaka, K.; Noi, K.; Baba, K.; Tsuda, H.; et al. Ultrasonication-based rapid amplification of alpha-synuclein aggregates in cerebrospinal fluid. *Sci. Rep.* **2019**, *9*, 6001, doi:10.1038/s41598-019-42399-0.
84. Kang, U.J.; Boehme, A.K.; Fairfoul, G.; Shahnawaz, M.; Ma, T.C.; Hutten, S.J.; Green, A.; Soto, C. Comparative study of cerebrospinal fluid alpha-synuclein seeding aggregation assays for diagnosis of Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2019**, *34*, 536–544, doi:10.1002/mds.27646.
85. Li, Q.X.; Mok, S.S.; Laughton, K.M.; McLean, C.A.; Cappai, R.; Masters, C.L.; Culvenor, J.G.; Horne, M.K. Plasma alpha-synuclein is decreased in subjects with Parkinson's disease. *Exp. Neurol.* **2007**, *204*, 583–588, doi:10.1016/j.expneurol.2006.12.006.
86. Shi, M.; Zabetian, C.P.; Hancock, A.M.; Ginchina, C.; Hong, Z.; Yearout, D.; Chung, K.A.; Quinn, J.F.; Peskind, E.R.; Galasko, D.; et al. Significance and confounders of peripheral DJ-1 and alpha-synuclein in Parkinson's disease. *Neurosci. Lett.* **2010**, *480*, 78–82, doi:10.1016/j.neulet.2010.06.009.
87. Wu, X.; Zheng, T.; Zhang, B. Exosomes in Parkinson's Disease. *Neurosci. Bull.* **2017**, *33*, 331–338, doi:10.1007/s12264-016-0092-z.
88. Yoo, Y.K.; Lee, J.; Kim, H.; Hwang, K.S.; Yoon, D.S.; Lee, J.H. Toward Exosome-Based Neuronal Diagnostic Devices. *Micromachines* **2018**, *9*, 634, doi:10.3390/mi9120634.
89. Xilouri, M.; Brekk, O.R.; Stefanis, L. alpha-Synuclein and protein degradation systems: A reciprocal relationship. *Mol. Neurobiol.* **2013**, *47*, 537–551, doi:10.1007/s12035-012-8341-2.
90. Zhao, Z.H.; Chen, Z.T.; Zhou, R.L.; Zhang, X.; Ye, Q.Y.; Wang, Y.Z. Increased DJ-1 and alpha-Synuclein in Plasma Neural-Derived Exosomes as Potential Markers for Parkinson's Disease. *Front. Aging Neurosci.* **2018**, *10*, 438, doi:10.3389/fnagi.2018.00438.
91. Bougea, A. If not salivary alpha-synuclein, then what? A look at potential Parkinson's disease biomarkers. *Expert Rev. Mol. Diagn.* **2020**, *20*, 359–361, doi:10.1080/14737159.2020.1721283.
92. Bougea, A.; Koros, C.; Stefanis, L. Salivary alpha-synuclein as a biomarker for Parkinson's disease: A systematic review. *J. Neural Transm. (Vienna, Austria: 1996)* **2019**, *126*, 1373–1382, doi:10.1007/s00702-019-02062-4.
93. Braak, H.; Del Tredici, K.; Rub, U.; de Vos, R.A.; Jansen Steur, E.N.; Braak, E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* **2003**, *24*, 197–211, doi:10.1016/s0197-4580(02)00065-9.
94. Ulusoy, A.; Rusconi, R.; Perez-Revuelta, B.I.; Musgrove, R.E.; Helwig, M.; Winzen-Reichert, B.; Di Monte, D.A. Caudo-rostral brain spreading of alpha-synuclein through vagal connections. *EMBO Mol. Med.* **2013**, *5*, 1119–1127, doi:10.1002/emmm.201302475.
95. Hilton, D.; Stephens, M.; Kirk, L.; Edwards, P.; Potter, R.; Zajicek, J.; Broughton, E.; Hagan, H.; Carroll, C. Accumulation of alpha-synuclein in the bowel of patients in the pre-clinical phase of Parkinson's disease. *Acta Neuropathol.* **2014**, *127*, 235–241, doi:10.1007/s00401-013-1214-6.
96. Shannon, K.M.; Keshavarzian, A.; Mutlu, E.; Dodiya, H.B.; Daian, D.; Jaglin, J.A.; Kordower, J.H. Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2012**, *27*, 709–715, doi:10.1002/mds.23838.
97. Stokholm, M.G.; Danielsen, E.H.; Hamilton-Dutoit, S.J.; Borghammer, P. Pathological alpha-synuclein in gastrointestinal tissues from prodromal Parkinson disease patients. *Ann. Neurol.* **2016**, *79*, 940–949, doi:10.1002/ana.24648.
98. Funayama, M.; Hasegawa, K.; Kowa, H.; Saito, M.; Tsuji, S.; Obata, F. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. *Ann. Neurol.* **2002**, *51*, 296–301, doi:10.1002/ana.10113.
99. Paisan-Ruiz, C.; Jain, S.; Evans, E.W.; Gilks, W.P.; Simon, J.; van der Brug, M.; Lopez de Munain, A.; Aparicio, S.; Gil, A.M.; Khan, N.; et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* **2004**, *44*, 595–600, doi:10.1016/j.neuron.2004.10.023.
100. Zimprich, A.; Biskup, S.; Leitner, P.; Lichtner, P.; Farrer, M.; Lincoln, S.; Kachergus, J.; Hulihan, M.; Uitti, R.J.; Calne, D.B.; et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* **2004**, *44*, 601–607, doi:10.1016/j.neuron.2004.11.005.
101. Smith, W.W.; Pei, Z.; Jiang, H.; Moore, D.J.; Liang, Y.; West, A.B.; Dawson, V.L.; Dawson, T.M.; Ross, C.A. Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 18676–18681, doi:10.1073/pnas.0508052102.

102. Greggio, E.; Jain, S.; Kingsbury, A.; Bandopadhyay, R.; Lewis, P.; Kaganovich, A.; van der Brug, M.P.; Beilina, A.; Blackinton, J.; Thomas, K.J.; et al. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol. Dis.* **2006**, *23*, 329–341.
103. Smith, W.W.; Pei, Z.; Jiang, H.; Dawson, V.L.; Dawson, T.M.; Ross, C.A. Kinase activity of mutant LRRK2 mediates neuronal toxicity. *Nat. Neurosci.* **2006**, *9*, 1231–1233, doi:10.1038/nn1776.
104. Daher, J.P.; Abdelmotilib, H.A.; Hu, X.; Volpicelli-Daley, L.A.; Moehle, M.S.; Fraser, K.B.; Needle, E.; Chen, Y.; Steyn, S.J.; Galatsis, P.; et al. Leucine-rich Repeat Kinase 2 (LRRK2) Pharmacological Inhibition Abates alpha-Synuclein Gene-induced Neurodegeneration. *J. Biol. Chem.* **2015**, *290*, 19433–19444, doi:10.1074/jbc.M115.660001.
105. Liu, Z.; Hamamichi, S.; Lee, B.D.; Yang, D.; Ray, A.; Caldwell, G.A.; Caldwell, K.A.; Dawson, T.M.; Smith, W.W.; Dawson, V.L. Inhibitors of LRRK2 kinase attenuate neurodegeneration and Parkinson-like phenotypes in *Caenorhabditis elegans* and *Drosophila* Parkinson's disease models. *Hum. Mol. Genet.* **2011**, *20*, 3933–3942, doi:10.1093/hmg/ddr312.
106. Deng, X.; Dzamko, N.; Prescott, A.; Davies, P.; Liu, Q.; Yang, Q.; Lee, J.D.; Patricelli, M.P.; Nomanbhoy, T.K.; Alessi, D.R.; et al. Characterization of a selective inhibitor of the Parkinson's disease kinase LRRK2. *Nat. Chem. Biol.* **2011**, *7*, 203–205, doi:10.1038/nchembio.538.
107. Ramsden, N.; Perrin, J.; Ren, Z.; Lee, B.D.; Zinn, N.; Dawson, V.L.; Tam, D.; Bova, M.; Lang, M.; Drewes, G.; et al. Chemoproteomics-based design of potent LRRK2-selective lead compounds that attenuate Parkinson's disease-related toxicity in human neurons. *ACS Chem. Biol.* **2011**, *6*, 1021–1028, doi:10.1021/cb2002413.
108. Fuji, R.N.; Flagella, M.; Baca, M.; Baptista, M.A.; Brodbeck, J.; Chan, B.K.; Fiske, B.K.; Honigberg, L.; Jubbe, A.M.; Katavolos, P.; et al. Effect of selective LRRK2 kinase inhibition on nonhuman primate lung. *Sci. Transl. Med.* **2015**, *7*, 273ra215, doi:10.1126/scitranslmed.aaa3634.
109. Tong, Y.; Yamaguchi, H.; Giaime, E.; Boyle, S.; Kopan, R.; Kelleher, R.J., 3rd; Shen, J. Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 9879–9884, doi:10.1073/pnas.1004676107.
110. Baptista, M.A.S.; Merchant, K.; Barrett, T.; Bhargava, S.; Bryce, D.K.; Ellis, J.M.; Estrada, A.A.; Fell, M.J.; Fiske, B.K.; Fuji, R.N.; et al. LRRK2 inhibitors induce reversible changes in nonhuman primate lungs without measurable pulmonary deficits. *Sci. Transl. Med.* **2020**, *12*, doi:10.1126/scitranslmed.aav0820.
111. Gilks, W.P.; Abou-Sleiman, P.M.; Gandhi, S.; Jain, S.; Singleton, A.; Lees, A.J.; Shaw, K.; Bhatia, K.P.; Bonifati, V.; Quinn, N.P.; et al. A common LRRK2 mutation in idiopathic Parkinson's disease. *Lancet* **2005**, *365*, 415–416, doi:10.1016/S0140-6736(05)17830-1.
112. Zhao, H.T.; John, N.; Delic, V.; Ikeda-Lee, K.; Kim, A.; Weihofen, A.; Swayze, E.E.; Kordasiewicz, H.B.; West, A.B.; Volpicelli-Daley, L.A. LRRK2 Antisense Oligonucleotides Ameliorate alpha-Synuclein Inclusion Formation in a Parkinson's Disease Mouse Model. *Mol. Ther. Nucleic Acids* **2017**, *8*, 508–519, doi:10.1016/j.omtn.2017.08.002.
113. Kluss, J.H.; Conti, M.M.; Kaganovich, A.; Beilina, A.; Melrose, H.L.; Cookson, M.R.; Mamais, A. Detection of endogenous S1292 LRRK2 autophosphorylation in mouse tissue as a readout for kinase activity. *NPJ Parkinson's Dis.* **2018**, *4*, 13, doi:10.1038/s41531-018-0049-1.
114. Sheng, Z.; Zhang, S.; Bustos, D.; Kleinheinz, T.; Le Pichon, C.E.; Dominguez, S.L.; Solanoy, H.O.; Drummond, J.; Zhang, X.; Ding, X.; et al. Ser1292 autophosphorylation is an indicator of LRRK2 kinase activity and contributes to the cellular effects of PD mutations. *Sci. Transl. Med.* **2012**, *4*, 164ra161, doi:10.1126/scitranslmed.3004485.
115. Dzamko, N.; Inesta-Vaquera, F.; Zhang, J.; Xie, C.; Cai, H.; Arthur, S.; Tan, L.; Choi, H.; Gray, N.; Cohen, P.; et al. The IkappaB kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during Toll-like receptor signaling. *PLoS ONE* **2012**, *7*, e39132, doi:10.1371/journal.pone.0039132.
116. Russo, I.; Berti, G.; Plotegher, N.; Bernardo, G.; Filograna, R.; Bubacco, L.; Greggio, E. Leucine-rich repeat kinase 2 positively regulates inflammation and down-regulates NF-kappaB p50 signaling in cultured microglia cells. *J. Neuroinflamm.* **2015**, *12*, 230, doi:10.1186/s12974-015-0449-7.
117. Fraser, K.B.; Moehle, M.S.; Alcalay, R.N.; West, A.B.; Consortium, L.C. Urinary LRRK2 phosphorylation predicts parkinsonian phenotypes in G2019S LRRK2 carriers. *Neurology* **2016**, *86*, 994–999, doi:10.1212/WNL.0000000000002436.

118. Fraser, K.B.; Moehle, M.S.; Daher, J.P.; Webber, P.J.; Williams, J.Y.; Stewart, C.A.; Yacoubian, T.A.; Cowell, R.M.; Dokland, T.; Ye, T.; et al. LRRK2 secretion in exosomes is regulated by 14-3-3. *Hum. Mol. Genet.* **2013**, *22*, 4988–5000, doi:10.1093/hmg/ddt346.
119. Fraser, K.B.; Rawlins, A.B.; Clark, R.G.; Alcalay, R.N.; Standaert, D.G.; Liu, N.; Parkinson's Disease Biomarker Program, C.; West, A.B. Ser(P)-1292 LRRK2 in urinary exosomes is elevated in idiopathic Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2016**, *31*, 1543–1550, doi:10.1002/mds.26686.
120. Ho, D.H.; Yi, S.; Seo, H.; Son, I.; Seol, W. Increased DJ-1 in urine exosome of Korean males with Parkinson's disease. *BioMed Res. Int.* **2014**, *2014*, 704678, doi:10.1155/2014/704678.
121. Cook, D.A.; Kannarkat, G.T.; Cintron, A.F.; Butkovich, L.M.; Fraser, K.B.; Chang, J.; Grigoryan, N.; Factor, S.A.; West, A.B.; Boss, J.M.; et al. LRRK2 levels in immune cells are increased in Parkinson's disease. *NPJ Parkinson's Dis.* **2017**, *3*, 11, doi:10.1038/s41531-017-0010-8.
122. Fan, Y.; Howden, A.J.M.; Sarhan, A.R.; Lis, P.; Ito, G.; Martinez, T.N.; Brockmann, K.; Gasser, T.; Alessi, D.R.; Sammler, E.M. Interrogating Parkinson's disease LRRK2 kinase pathway activity by assessing Rab10 phosphorylation in human neutrophils. *Biochem. J.* **2018**, *475*, 23–44, doi:10.1042/bcj20170803.
123. Dzamko, N.; Chua, G.; Ranola, M.; Rowe, D.B.; Halliday, G.M. Measurement of LRRK2 and Ser910/935 phosphorylated LRRK2 in peripheral blood mononuclear cells from idiopathic Parkinson's disease patients. *J. Parkinson's Dis.* **2013**, *3*, 145–152, doi:10.3233/jpd-130174.
124. Thevenet, J.; Pescini Gobert, R.; Hooft van Huijsduijnen, R.; Wiessner, C.; Sagot, Y.J. Regulation of LRRK2 expression points to a functional role in human monocyte maturation. *PLoS ONE* **2011**, *6*, e21519, doi:10.1371/journal.pone.0021519.
125. Padmanabhan, S.; Lanz, T.A.; Gorman, D.; Wolfe, M.; Joyce, A.; Cabrera, C.; Lawrence-Henderson, R.; Levers, N.; Joshi, N.; Ma, T.; et al. An Assessment of LRRK2 Serine 935 Phosphorylation in Human Peripheral Blood Mononuclear Cells in Idiopathic Parkinson's Disease and G2019S LRRK2 Cohorts. *J. Parkinson's Dis.* **2020**, doi:10.3233/jpd-191786.
126. Thirstrup, K.; Dachsel, J.C.; Oppermann, F.S.; Williamson, D.S. Selective LRRK2 kinase inhibition reduces phosphorylation of endogenous Rab10 and Rab12 in human peripheral mononuclear blood cells. *Sci. Rep.* **2017**, *7*, 10300, doi:10.1038/s41598-017-10501-z.
127. Atashrazm, F.; Hammond, D.; Perera, G.; Bolliger, M.F.; Matar, E.; Halliday, G.M.; Schule, B.; Lewis, S.J.G.; Nichols, R.J.; Dzamko, N. LRRK2-mediated Rab10 phosphorylation in immune cells from Parkinson's disease patients. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2019**, *34*, 406–415, doi:10.1002/mds.27601.
128. Wang, S.; Liu, Z.; Ye, T.; Mabrouk, O.S.; Maltbie, T.; Aasly, J.; West, A.B. Elevated LRRK2 autophosphorylation in brain-derived and peripheral exosomes in LRRK2 mutation carriers. *Acta Neuropathol. Commun.* **2017**, *5*, 86, doi:10.1186/s40478-017-0492-y.
129. Perera, G.; Ranola, M.; Rowe, D.B.; Halliday, G.M.; Dzamko, N. Inhibitor treatment of peripheral mononuclear cells from Parkinson's disease patients further validates LRRK2 dephosphorylation as a pharmacodynamic biomarker. *Sci. Rep.* **2016**, *6*, 31391, doi:10.1038/srep31391.
130. Nichols, R.J.; Dzamko, N.; Morrice, N.A.; Campbell, D.G.; Deak, M.; Ordureau, A.; Macartney, T.; Tong, Y.; Shen, J.; Prescott, A.R.; et al. 14-3-3 binding to LRRK2 is disrupted by multiple Parkinson's disease-associated mutations and regulates cytoplasmic localization. *Biochem. J.* **2010**, *430*, 393–404, doi:10.1042/bj20100483.
131. Dzamko, N.; Deak, M.; Hentati, F.; Reith, A.D.; Prescott, A.R.; Alessi, D.R.; Nichols, R.J. Inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser(910)/Ser(935), disruption of 14-3-3 binding and altered cytoplasmic localization. *Biochem. J.* **2010**, *430*, 405–413.
132. Harvey, K.; Outeiro, T.F. The role of LRRK2 in cell signalling. *Biochem. Soc. Trans.* **2019**, *47*, 197–207, doi:10.1042/bst20180464.
133. Ito, G.; Katsemonova, K.; Tonelli, F.; Lis, P.; Baptista, M.A.; Shpiro, N.; Duddy, G.; Wilson, S.; Ho, P.W.; Ho, S.L.; et al. Phos-tag analysis of Rab10 phosphorylation by LRRK2: A powerful assay for assessing kinase function and inhibitors. *Biochem. J.* **2016**, *473*, 2671–2685, doi:10.1042/bcj20160557.
134. Pfeffer, S.R. Rab GTPases: Master regulators that establish the secretory and endocytic pathways. *Mol. Biol. Cell* **2017**, *28*, 712–715, doi:10.1091/mbc.E16-10-0737.
135. Steger, M.; Tonelli, F.; Ito, G.; Davies, P.; Trost, M.; Vetter, M.; Wachter, S.; Lorentzen, E.; Duddy, G.; Wilson, S.; et al. Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a subset of Rab GTPases. *eLife* **2016**, *5*, e12813, doi:10.7554/eLife.12813.

136. Rieckmann, J.C.; Geiger, R.; Hornburg, D.; Wolf, T.; Kveler, K.; Jarrossay, D.; Sallusto, F.; Shen-Orr, S.S.; Lanzavecchia, A.; Mann, M.; et al. Social network architecture of human immune cells unveiled by quantitative proteomics. *Nat. Immunol.* **2017**, *18*, 583–593, doi:10.1038/ni.3693.
137. Konoshenko, M.Y.; Lekchnov, E.A. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *BioMed Res. Int.* **2018**, *2018*, 8545347, doi:10.1155/2018/8545347.
138. Li, P.; Kaslan, M.; Lee, S.H.; Yao, J.; Gao, Z. Progress in Exosome Isolation Techniques. *Theranostics* **2017**, *7*, 789–804, doi:10.7150/thno.18133.
139. Wang, S.; West, A.B. Caught in the act: LRRK2 in exosomes. *Biochem. Soc. Trans.* **2019**, *47*, 663–670, doi:10.1042/bst20180467.
140. Gomez, R.C.; Wawro, P. Membrane association but not identity is required for LRRK2 activation and phosphorylation of Rab GTPases. *J. Cell Biol.* **2019**, *218*, 4157–4170, doi:10.1083/jcb.201902184.
141. Liu, Z.; Bryant, N.; Kumaran, R.; Beilina, A.; Abeliovich, A.; Cookson, M.R.; West, A.B. LRRK2 phosphorylates membrane-bound Rabs and is activated by GTP-bound Rab7L1 to promote recruitment to the trans-Golgi network. *Hum. Mol. Genet.* **2018**, *27*, 385–395, doi:10.1093/hmg/ddx410.
142. Purlyte, E.; Dhekne, H.S.; Sarhan, A.R.; Gomez, R.; Lis, P.; Wightman, M.; Martinez, T.N.; Tonelli, F.; Pfeffer, S.R.; Alessi, D.R. Rab29 activation of the Parkinson's disease-associated LRRK2 kinase. *EMBO J.* **2019**, *38*, doi:10.15252/embj.2018101237.
143. Lwin, A.; Orvisky, E.; Goker-Alpan, O.; LaMarca, M.E.; Sidransky, E. Glucocerebrosidase mutations in subjects with parkinsonism. *Mol. Genet. Metab.* **2004**, *81*, 70–73, doi:10.1016/j.ymgme.2003.11.004.
144. Neudorfer, O.; Giladi, N.; Elstein, D.; Abrahamov, A.; Turezkite, T.; Aghai, E.; Reches, A.; Bembi, B.; Zimran, A. Occurrence of Parkinson's syndrome in type I Gaucher disease. *QJM: Mon. J. Assoc. Physicians* **1996**, *89*, 691–694, doi:10.1093/qjmed/89.9.691.
145. Sidransky, E.; Nalls, M.A.; Aasly, J.O.; Aharon-Peretz, J.; Annesi, G.; Barbosa, E.R.; Bar-Shira, A.; Berg, D.; Bras, J.; Brice, A.; et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N. Engl. J. Med.* **2009**, *361*, 1651–1661, doi:10.1056/NEJMoa0901281.
146. Neumann, J.; Bras, J.; Deas, E.; O'Sullivan, S.S.; Parkkinen, L.; Lachmann, R.H.; Li, A.; Holton, J.; Guerreiro, R.; Paudel, R.; et al. Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. *Brain: A J. Neurol.* **2009**, *132*, 1783–1794, doi:10.1093/brain/awp044.
147. Gegg, M.E.; Burke, D.; Heales, S.J.; Cooper, J.M.; Hardy, J.; Wood, N.W.; Schapira, A.H. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. *Ann. Neurol.* **2012**, *72*, 455–463, doi:10.1002/ana.23614.
148. Cilia, R.; Tunesi, S.; Marotta, G.; Cereda, E.; Siri, C.; Tesi, S.; Zecchinelli, A.L.; Canesi, M.; Mariani, C.B.; Meucci, N.; et al. Survival and dementia in GBA-associated Parkinson's disease: The mutation matters. *Ann. Neurol.* **2016**, *80*, 662–673, doi:10.1002/ana.24777.
149. Koros, C.; Simitsi, A.; Stefanis, L. Genetics of Parkinson's Disease: Genotype-Phenotype Correlations. *Int. Rev. Neurobiol.* **2017**, *132*, 197–231, doi:10.1016/bs.irm.2017.01.009.
150. Mazzulli, J.R.; Xu, Y.H.; Sun, Y.; Knight, A.L.; McLean, P.J.; Caldwell, G.A.; Sidransky, E.; Grabowski, G.A.; Krainc, D. Gaucher disease glucocerebrosidase and alpha-synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell* **2011**, *146*, 37–52, doi:10.1016/j.cell.2011.06.001.
151. Sardi, S.P.; Cheng, S.H.; Shihabuddin, L.S. Gaucher-related synucleinopathies: The examination of sporadic neurodegeneration from a rare (disease) angle. *Prog. Neurobiol.* **2015**, *125*, 47–62, doi:10.1016/j.pneurobio.2014.12.001.
152. Alcalay, R.N.; Levy, O.A.; Waters, C.C.; Fahn, S.; Ford, B.; Kuo, S.H.; Mazzoni, P.; Pauciulo, M.W.; Nichols, W.C.; Gan-Or, Z.; et al. Glucocerebrosidase activity in Parkinson's disease with and without GBA mutations. *Brain: A J. Neurol.* **2015**, *138*, 2648–2658, doi:10.1093/brain/awv179.
153. Sardi, S.P.; Clarke, J.; Viel, C.; Chan, M.; Tamsett, T.J.; Treleaven, C.M.; Bu, J.; Sweet, L.; Passini, M.A.; Dodge, J.C.; et al. Augmenting CNS glucocerebrosidase activity as a therapeutic strategy for parkinsonism and other Gaucher-related synucleinopathies. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 3537–3542, doi:10.1073/pnas.1220464110.
154. Migdalska-Richards, A.; Daly, L.; Bezard, E.; Schapira, A.H. Ambroxol effects in glucocerebrosidase and alpha-synuclein transgenic mice. *Ann. Neurol.* **2016**, *80*, 766–775, doi:10.1002/ana.24790.
155. Mullin, S.; Smith, L.; Lee, K.; D'Souza, G.; Woodgate, P.; Elflein, J.; Hallqvist, J.; Toffoli, M.; Streeter, A.; Hosking, J.; et al. Ambroxol for the Treatment of Patients with Parkinson Disease with and Without

- Glucocerebrosidase Gene Mutations: A Nonrandomized, Noncontrolled Trial. *JAMA Neurol.* **2020**, *77*, 427–434, doi:10.1001/jamaneurol.2019.4611.
156. Papagiannakis, N.; Xilouri, M.; Koros, C.; Stamelou, M.; Antonelou, R.; Maniati, M.; Papadimitriou, D.; Moraitou, M.; Michelakakis, H.; Stefanis, L. Lysosomal alterations in peripheral blood mononuclear cells of Parkinson's disease patients. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2015**, *30*, 1830–1834, doi:10.1002/mds.26433.
 157. Pchelina, S.; Emelyanov, A.; Baydakova, G.; Andoskin, P.; Senkevich, K.; Nikolaev, M.; Miliukhina, I.; Yakimovskii, A.; Timofeeva, A.; Fedotova, E.; et al. Oligomeric alpha-synuclein and glucocerebrosidase activity levels in GBA-associated Parkinson's disease. *Neurosci. Lett.* **2017**, *636*, 70–76, doi:10.1016/j.neulet.2016.10.039.
 158. Xicoy, H.; Penuelas, N.; Vila, M.; Laguna, A. Autophagic- and Lysosomal-Related Biomarkers for Parkinson's Disease: Lights and Shadows. *Cells* **2019**, *8*, doi:10.3390/cells8111317.
 159. Cerri, S.; Ghezzi, C.; Sampieri, M.; Siani, F.; Avenali, M.; Dornini, G.; Zangaglia, R.; Minafra, B.; Blandini, F. The Exosomal/Total alpha-Synuclein Ratio in Plasma Is Associated WITH Glucocerebrosidase Activity and Correlates With Measures of Disease Severity in PD Patients. *Front. Cell. Neurosci.* **2018**, *12*, 125, doi:10.3389/fncel.2018.00125.
 160. Parnetti, L.; Paciotti, S.; Eusebi, P.; Dardis, A.; Zampieri, S.; Chiasserini, D.; Tasegian, A.; Tambasco, N.; Bembi, B.; Calabresi, P.; et al. Cerebrospinal fluid beta-glucocerebrosidase activity is reduced in parkinson's disease patients. *Mov. Disord. Off. J. Mov. Disord. Soc.* **2017**, *32*, 1423–1431, doi:10.1002/mds.27136.
 161. Parnetti, L.; Chiasserini, D.; Persichetti, E.; Eusebi, P.; Varghese, S.; Qureshi, M.M.; Dardis, A.; Deganuto, M.; De Carlo, C.; Castrioto, A.; et al. Cerebrospinal fluid lysosomal enzymes and alpha-synuclein in Parkinson's disease. *Mov. Disord.: Off. J. Mov. Disord. Soc.* **2014**, *29*, 1019–1027, doi:10.1002/mds.25772.
 162. Kestenbaum, M.; Alcalay, R.N. Clinical Features of LRRK2 Carriers with Parkinson's Disease. *Adv. Neurobiol.* **2017**, *14*, 31–48, doi:10.1007/978-3-319-49969-7_2.
 163. Zhu, X.; Babar, A.; Siedlak, S.L.; Yang, Q.; Ito, G.; Iwatsubo, T.; Smith, M.A.; Perry, G.; Chen, S.G. LRRK2 in Parkinson's disease and dementia with Lewy bodies. *Mol. Neurodegener.* **2006**, *1*, 17, doi:10.1186/1750-1326-1-17.
 164. Di Maio, R.; Hoffman, E.K.; Rocha, E.M.; Keeney, M.T.; Sanders, L.H.; De Miranda, B.R.; Zharikov, A.; Van Laar, A.; Stepan, A.F.; Lanz, T.A.; et al. LRRK2 activation in idiopathic Parkinson's disease. *Sci. Transl. Med.* **2018**, *10*, eaar5429, doi:10.1126/scitranslmed.aar5429.
 165. Shihabuddin, L.S.; Brundin, P.; Greenamyre, J.T.; Stephenson, D.; Sardi, S.P. New Frontiers in Parkinson's Disease: From Genetics to the Clinic. *J. Neurosci.* **2018**, *38*, 9375–9382, doi:10.1523/JNEUROSCI.1666-18.2018.
 166. Johnson, M.E.; Stecher, B.; Labrie, V.; Brundin, L.; Brundin, P. Triggers, Facilitators, and Aggravators: Redefining Parkinson's Disease Pathogenesis. *Trends Neurosci.* **2019**, *42*, 4–13, doi:10.1016/j.tins.2018.09.007.
 167. Bordet, R.; Ihl, R.; Korczyn, A.D.; Lanza, G.; Jansa, J.; Hoerr, R.; Guekht, A. Towards the concept of disease-modifier in post-stroke or vascular cognitive impairment: A consensus report. *BMC Med.* **2017**, *15*, 107, doi:10.1186/s12916-017-0869-6.
 168. Vinciguerra, L.; Lanza, G.; Puglisi, V.; Fisicaro, F.; Pennisi, M.; Bella, R.; Cantone, M. Update on the Neurobiology of Vascular Cognitive Impairment: From Lab to Clinic. *Int. J. Mol. Sci.* **2020**, *21*, doi:10.3390/ijms21082977.
 169. Manzoni, C.; Lewis, P.A. Dysfunction of the autophagy/lysosomal degradation pathway is a shared feature of the genetic synucleinopathies. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **2013**, *27*, 3424–3429, doi:10.1096/fj.12-223842.

